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13. ABSTRACT *(Maximum 200 words)*

Studies during the ongoing grant period demonstrated that $CD4^+$ cells specific for the HER-2 helper epitope G89 (777-789) are present in an activated form in healthy donors and cancer patients. G89-reactive cells, rapidly induce IL-12 from antigen presenting cells (dendritic cells). DC induces CD40 upregulation on DC. They are also necessary and required for induction of specific CTL activity to a + specific CTL epitope. However, G89-reactive $CD4^+$ cells show significantly weaker reactivity with G89 in the lymph nodes containing breast cancer cells. Simultaneous presentation of a HER-2 CTL epitope, and of a HER-2 helper epitope leads to higher levels of IFN-γ than presentation of each epitope alone. Since IFN-γ is downmodulator of HER-2 and effector molecule in inhibiting the growth of HER-2^+ breast cancer cells, its induction in situ may have beneficial effects for controlling the disease.

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Abbreviations used: Cytotoxic T lymphocytes, CTL; Peripheral blood mononuclear cells, PBMC; Antigen, Ag; T cell receptor, TCR; HER-2/neu proto-oncogene, HER-2; stimulation index, S.I.; standard deviation, SD; tetanus toxoid, TT; influenza hemaglutinin, HA; wild-type, w.t.; lymph node, LN, dendritic cells, DC, T helper 1, Th1, T helper 2, Th2, CD40, cluster of differentiation 40, (surface antigen, gp39 receptor). CD40 ligand (gp39, CD154), Mean Channel Fluorescence, MCF.

INTRODUCTION

The overall objective of these studies is to develop novel therapeutic approaches to breast cancer by understanding the requirements for successful induction of anti-tumor responses using newly defined T cell epitopes. The use of specific epitopes can overcome limitations in the use of whole self-protein or biased involvement of Th2 cells. On the other hand, the whole protein is not sufficient for activation of immune cells (1). Breast tumors release tumor Ag in the environment, while antigen presenting cells (APC) macrophages, MØ, dendritic cells (DC) uptake and re-present tumor proteins. However the disease progresses indicating that this response is inefficient or suppressed. Our recent studies identified an immunodominant CTL epitope on HER-2(2). Furthermore, we identified immunodominant epitopes on two other tumor proteins: Folate binding protein/Folate receptor α(FBP/FR-α) and the aminoenhancer of split (AES) of the Notch complex (3,4). These epitopes by themselves cannot induce a CTL response to the Ag and/or tumor. For this reason we are currently using the Th1 subset of $CD4^+$ cells to induce and amplify a CTL response to tumor. The rationale and the reasons for the use of the helper epitopes have been described in detail in the previous report and are not repeated here.

Furthermore, studies during the previous year have identified a dominant HER-2 epitope recognized by $CD4^+$ cells designated as G89 = HER-2 (777-790) SPYVSRLL GICL. This epitope induced proliferation and type 1 cytokines from PBMC of breast cancer patients, and healthy donors, suggesting that precursors for HER-2 specific Th1 $CD4^+$ cells exist (5). To address the role of the $CD4^+$ epitope G89 and of its related epitope F7 (HER-2, 776-789) (5,6) in specific cell mediated ummunity (7), our studies, during this period focussed first on characterization of the interaction between Th1 cells and APC. This aimed to address whether interaction of Th1 with APC induced expression of B7 and/or of other costimulatory molecules as well.

A second objective of the studies of the interaction between APC and Th1 used was to determine whether this interaction induces predominantly inflamatory cytokines from DC (i.e. Interleukin-12, IL-12), with reciprocal induction of IFN-γ from T cells. Conditioning APC for activation (IL-12, IFN-γ) versus tolerance/suppression (no IL-12, but IL-10) (8,9,10,11) is an indication of the stimulatory ability of $CD4^+$ cells, and of their function as Th1 cells. (7)

A third objective of this study was to determine the ability of the newly identified immunodominant CTL and Th epitopes to induce a CTL response or to enhance an existent CTL response. A fourth objective of this study was to determine whether HER-2 peptides with Th1 activity can activate T cells during disease progression.

We found that G89 recognition of APC(DC) enhanced expression of CD40, suggesting a role for the Th1 cells interacting with DC in the induction of IL-12 $\rightarrow$ IFN-γ pathway. We also found that interaction of Th1 cells with DC during recognition of G89 induces IL-12 from DC and IFN-γ from Th1 cells. Thus recognition of Th1 HER-2 peptides conditioned APC for an inflamatory (autoimmune response). A third finding was that G89 is required for induction of E75 specific CTL. However, in lymph nodes with disease, stimulation with G89 followed by culture in IL-2, and restimulation of the resulting cells did not induce G89-specific cells expansion. Our results suggest that the lack of G89 response during disease progression may be associated with failure of an immune response to breast cancer. Breast cancer progresses through lymph nodes. Thus our results identified a key point where optimization of the immune response to breast cancer should be targeted.

MATERIALS AND METHODS

Cells. HLA-A2$^+$ PBMC were obtained from healthy volunteers from the Blood Bank of M.D. Anderson Cancer Center. T2 cells, ovarian SKOV3, SKOV3.A2 cells, and indicator tumors from ovarian ascites were described (2).

Antibodies and Cytokines. mAb to CD3, CD4, CD8 (Ortho), CD13 and CD14 (Caltag Laboratories, San Francisco, CA), B7.1 and B7.2 (CD80 and CD86, Calbiochem), ICAM-1 (CD54, Calbiochem), CD40L (Ancell, Bayport, MN), HLA-A2 (clone BB7.2, ATCC), and MHC-II (L243, DAKO Corp., Carpintera, CA) were used as unconjugated FITC or PE conjugated. Anti-CTLA-4 was a kind gift from Dr. Peter Linsley (Bristol-Myers). mAb specific for IL-12, IFN-γ and isotype controls were obtained from Pharmingen. The following cytokines were used: GM-CSF (Immunex Corp., Washington, DC), specific activity 1.25 x 10^7 CFU/250 mg; TNF- (Cetus Corp., Emeryville, CA), specific activity 2.5 x 10^7 U/mg IL-4 (Biosource International), specific activity, 2 x 10^6 U/mg; IL-2 (Cetus), specific activity 18 x 10^6 IU/mg; IL-12 of specific activity 5 x 10^6 U/mg was a kind gift from Dr. Stanley Wolf, Department of Immunology, Genetics Institute, Cambridge, MA.

Synthetic peptides. The HER-2 peptides used were: E75 (369-377); and the helper peptides: F7 (HER-2, 776-788): GSPYVSRLLGICL, F6: (HER-2, 776-793) GSPYVSRLLGICLTSTVQ. G89: (HER-2 777-789):SPYVSRLLGICLT. The modified Muc-1 peptides used were D125: (GVTSAKDTRV) and D132 (SLADPAHGV). The positive control CTL epitope used was the influenza matrix peptide (58-66): GILGFVFTL, designated as M1. M1 forms an immunodominant epitope recognized by memory CTL in healthy donors (12). All peptides were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center and purified by HPLC. Peptides were 95-97% pure by amino acid analysis. Peptides were dissolved in PBS and stored frozen at –20 °C in aliquots of 2 mg/ml.

Immunofluorescence. Antigen expression by DC, T2 cells, and T cells was determined by FACS using a flow-cytometer (EPICS - Profile Analyzer, Coulter Co, Hialeh FL). DC cells were defined by the presence of CD13 and absence of CD14 marker after culture in GM-CSF and IL-4. For phenotype analysis, DC were incubated with PE-conjugated anti CD13 mAb and FITC-conjugated mAb specific for a surface Ag. For determination of the effects of cytokines,

peptides and T cells on surface antigen expression, DC were incubated with the same amounts of cytokines and peptides as in T cell activation assays for 24 h, and the levels of Ag expression were determined in the gated CD13$^+$ population.

Culture of PBMC-derived DC. CD13$^+$ DC were generated from freshly isolated PBMC following the established CD14 methods (13, 14). Complete RPMI medium (containing 10% FCS) supplemented with 1000 U/ml GM-CSF or 500 U/ml IL-4 was added to each well containing plastic-adherent cells and maintained for 7 days. T cells were obtained from the plastic non-adherent PBMC by removal of CD16$^+$ and CD56$^+$ cells. CD8$^+$ cells were isolated by removing first the CD4$^+$, and then the CD16$^+$ and CD56$^+$ cells from the nonadherent population using Dynabeads (Dynal, Oslo, Norway). CD8$^+$ subpopulations were obtained using anti-CD45RO mAb and anti-CD45RA mAb (UCHL-1, DAKO) as described (12). After depletion, the resulting cells were 97% CD8$^+$ as determined by flow cytometry.

T cell stimulation by peptide pulsed DC.   We used CD13$^+$ DC, as APC because such cells have been reported to activate both naive and memory CTL ( ) and to present E75 (15, 16). After culture in GM-CSF+IL-4, DC from all donors were >96% CD13$^+$, and CD14$^-$ expressed high levels of MHC-I, MHC-II, CD54, CD40, and CD86, but lower levels of CD80 in agreement with the described phenotype of peripheral blood CD14$^+$ derived DC (12). DC were plated at 1.2 x 10^5 cell/well in 24-well culture plates, and pulsed with peptides at 50 μg/ml in serum-free medium for 4 h before addition of responders. TNF-$\propto$ (50 U/ml) was added to DC for the last hour to stimulate Ag uptake and presentation (13). Autologous, isolated CD8$^+$ cells or isolated CD8$^+$ cells (CD45RO$^+$ and CD45R0+cells depleted) in RPMI 1640 containing 10% HS were added to DC at 1.5 x 10^6/ml, followed by IL-12. IL-2 was added 12-16 h later to each well. For inhibition studies, mAb specific for B7.1, B7.2, HLA-A2 and isotype control MOPC myeloma were added to DC or tumor cells, 1 h before responders in amounts reported to be inhibitory by the manufacturers. Anti CTLA-4 and CD40L were added to T cells 1 h before they were added to cultures. The effects of peptides and cytokines on T cell survival were determined by counting the numbers of recovered viable cells, and determinating the numbers of CD8$^+$ and CD4$^+$ cells in the sample by flow-cytometry. Specific proliferative responses to E75 in the presence or absence of cytokines were determined by measuring the incorporated radioactivity in equal cell numbers pulsed with 1 μCi of (^{3}H)-TdR (5, 6).

CTL and cytokine assays. Recognition of peptides used as immunogens by CTL was performed as described (2). Equal numbers of viable effectors from each well were used in all assays. Supernatants collected at 6, 24, or 48 h were tested in duplicate for the presence of IL-2, IL-4, IL-10, IL-12 and IFN-γ using cytokine ELISA-kits -(Biosource International, Camarillo, CA) or R&D systems as described (6) with a sensitivity of 4-7 pg/ml. IL-12 was detected using an ELISA kit which recognizes both p40 and the natural heterodimeric molecule. The amount of cytokines was quantitated using standard plots of known concentrations of cytokines determined in the same experiment.

Proliferation assays. For proliferation assays 100 μl aliquots were removed from each well of the 24-well plate of primary cultures after 4-6 days as described (6). Tetraplicate samples were cultured in a 96 well plate with 1 μCi [^{3}H]-Tdr in a final volume of 200 μl. The cells were harvested 16 h later, and the radioactivity counted in a Beckman LS3501 liquid scintillation counter (6). A positive proliferative response was defined as positive when differences in cpm values between cultures that received peptides compared with cultures which did not receive peptides were significant by the unpaired Student's t-test ($p < 0.05$). Stimulation indexes (S.I.) represented the ratio between the mean c.p.m. of the cultures stimulated with peptide, and the mean c.p.m. of the cultures that have not been stimulated with peptide (N.P.).

Cytokine production. The ability of PBMC to secrete antigen-induced IFN-γ, IL–4, and IL-10 was determined by culturing the PBMC with the corresponding peptides. Supernatants were collected at different times and stored frozen at -20°C. The cytokine concentrations were measured by double sandwich-ELISA using the corresponding kits provided by BioSource International (Camariyo, CA). The cytokine assays were calibrated with human recombinant IFN-γ, IL-4, and IL-10 to detect each cytokine in the range of 15-1000 pg/ml.

Statistical Methods. Differences in proliferative responses were analyzed using Student's t-test for unpaired samples.

RESULTS

Characterization of recognition of Th1 peptide G89, presented on dendritic cells (DC) by T cells.

To characterize the interactions between CD4^{+} cells and APC and their effects on surface Ag expression and cytokine induction we focussed on peptide G89 (Tutle et. al., Clinical Cancer Res. 4:2015-2024, 1998) (5). Our studies show that G89 is recognized by the majority of HLA-DR4^{+} healthy donors and breast cancer patients with no evidence of disease (5). To identify the effects of G89 recognition on activation of antigen presenting cells (APC) we first generated dendritic cells (DC). DC are considered the most efficient APC, for activation of immune responses (13). Results from several laboratories indicate that DC have an excellent potential for cancer vaccination. We generated DC by culture of plastic-adherent PBMC in GM-CSF and IL-4 (described in details in the Materials and Methods section) DC generated from PBMC are characterized by the expression of the CD13 marker and dissappearance of the CD14 (monocyte marker).

To characterize the effects of G89 recognition on surface Ag expression on DC, DC were pulsed with G89 and the expression of CD13, CD40, MHC-I (W6/32), MHC-II, (HLA-DR) B7.1, and ICAM-1 was determined after costimulation in the presence or absence of freshly isolated autologous T cells. The results in **Fig. 1** show that incubation of DC with autologous T cells lead to upregulation of CD40, MHC-1 and MHC-II antigens but not of B7.1. This experiment was repeated with two additional donors and the results were confirmed.

To address whether this is the result of recognition of G89, T cells (CD8^{+}depleted) from the same donor were primed with G89 (designated as G89P). In a separate experiment isolated CD8^{+} cells were primed with E75, (E75P). Then autologous DC were incubated in parallel with G89P cells, with G89P cells + G89, with E75P + E75, or with (G89P + G89) together with (E75P + E75). 20 h later (to determine the early response), supernatants were collected and used for quantitation of the IFN-γ secreted, while DC were collected and the expression of CD40 was determined in the gated CD13^{+} population using two colors FACS. analysis. The results in **Fig. 2** show that recognition of G89P cells induced low levels of IFN-γ. These levels increased by two fold when G89 was added at 25 μg/ml. Similarly recognition of E75 on DC by E75P T cells induced IFN-γ. Simultaneous stimulation of G89P and E75P with DC pulsed with E75 + G89

demonstrated a synergistic effect of G89 and E75 in IFN-γ induction. Since IL-12, which is a costimulator of the IFN-γ induction in response to Ag (17), was not used, these results demonstrate a synergy Th1 + CD8$^+$ in IFN-γ induction.

Furthermore, recognition of G89 by G89P increased CD40 expression by more than 2.5 fold, confirming the results in **Fig. 1**. E75 + E75P had a much weaker effect on CD40 induction. Of interest when both peptides (E75 + G89) and G89P + E75P cells were used in the same experiment, the increase in CD40 expression was weaker than the increase induced by G89P alone. However E75 + E75P induced only a modest increase in CD40 expression, thus compared with the increase induced by the CTL epitope alone, G89 + G89P enhanced CD40 levels on APC.

These effects were followed by induction of CTL activity at priming. This experiment is described here together with CD40 and IFN-γ induction for improved clarity. CD8$^+$ cells from the same donor were stimulated with DC alone (DC) as a control, DC pulsed with E75 (DC-E75), DC pulsed with G89 (DC-G89), or DC-E75-G89, in the absence, or presence of G89P cells (in the G89 groups). The results are shown in **Fig. 3**. The CTL activity was determined in both 5 h and 20 h assays to detect the presence of even few E75 reacting CTL. The results show that E75 specific activity was detected only in the DC-G89-E75 group at 20h, suggesting that the E75-specific CTL were induced but the frequency of E75-specific cells was low. No specific CTL activity was detected in the other three groups, indicating that G89 is required for HER-2 (E75) - specific CTL induction.

E75 and G89 also appear to synergize in T cell proliferation and expansion. This is indicated by a parallel experiment where isolated T cells from the same donor (CD4 and CD8$^+$) were stimulated in the same conditions with E75, G89, E75 + G89, or as control E75 + D100. D100 is a HER-2 peptide with no proliferation induced activity. The results in **Fig. 4** show that stimulation of these cells with E75 + G89 has a strong synergistic effect in enhancing their expansion compared with stimulation with E75 or G89 alone.

HER-2 epitopes F7 and G89 are required for induction and/or expansion of CTL.

To address the question whether CD4$^+$ help is required for induction of CTL activity, we extended the studies presented above to stimulation of DC with T cell lines induced by stimulation with HER-2 peptides F7 and F13. Because of their potential for cross-reactivity, F7 and F13 are more suitable for stimulation of T cells from donors of phenotypes distinct from HLA-DR4. A F7-reactive T cell line was induced from a HLA-DR4$^+$ healthy donor. In this line CD4$^+$ cells were 76%, while CD8$^+$ cells were 18%. A CTL induction experiment was established to test the feasibility of the approach. DC were pulsed with E75 in the presence or absence of F7 in parallel groups. Autologous plastic non-adherent PBMC were used as responders. The autologous CD4$^+$ line was added at a ratio of helper to responder (1:10, i.e. 10^5 CD4$^+$ line cells, to 10^6 Auto-PBMC). To continue the investigation of the nature of the helper effects by recognition of Th peptides IL-12 was added in a parallel experiment. The results summarized in Table II show that E15 specific CTL activity was detected at priming when the CD4$^+$ line was added but required either the presence of F7 or in the presence of IL-12.

These results confirm previous results (p9, 10) regarding CD40 upregulation and IFN-γ induction. Since the T cell dependent pathway for IL-12 induction involve CD40-CD40L interactions (18, 19), these results suggest that CD4$^+$ cells/line recognition of G89/F7 on APC, activates APC.

The enhancing ability of HER-2 peptide G89 was also confirmed using as responders breast TIL. Since the MHC-II phenotype of these cells was not known, we stimulated these TIL with E75 + F13, or E75 + F7 using E75 as a control. The results in **Fig. 5**, show that in this case, F13 had a stronger stimulatory activity than F7. The CTL activity of TIL stimulated by E75 + F13 increased 5 fold compared with the TIL stimulated with E75 alone. F7 in this case had no effect. Thus helper HER-2 peptides are required both for priming and maintenance and expansion of a CTL response.

Since the preliminary experiments show that (a) CTL activation require CD4$^+$ cell help, which is mediated by G89, and (b) CTL activity is induced by stimulation with E75 + G89, we expanded on these experiments to establish the requirements for G89 in CTL generation.

Four groups were set in parellel:

(1)   Priming: DC-G89 with G89P + IL-12 : Restimulation : DC-575 E75P + IL-12

(2)   Priming: DC-G89 with G89P + IL12 : Restimulation : DC-E75-G89 E75P + G89P + IL-12

(3)   Priming: DC-G89 with G89P + IL-12 : Restimulation : DC-NP NP-P + IL-12

(4)   Priming: DC-NP with NP-P + IL-12 : Restimulation : DC-E75-G89 + E75P + G89P + IL-12

As responders plastic non-adherent T cells were used since they contained both CD4$^+$ and CD8$^+$ cells. The results are shown in **Fig. 6**. At this time highly specific CTL activity was observed only from the group (2) where G89P + G89 were included both in the priming and restimulation phases. The absence of G89 + G89P during priming (Group 4) or of G89P + G89 during restimulation (Group 1) lead to nonspecific CTL activity. These results indicate that for induction of E75-epitope specific CTL both E75 and G89 are required at the priming and restimulation phases. Since IL-12 was present in all groups, these results indicate that additional factors are involved in the helper effect mediated by G89.

Proliferative responses to G89 are inhibited in tumor positive lymph nodes.

To address the question whether G89 recognition is affected during disease progression, we continued our investigation of the responses to G89 of the T cells from tumor positive lymph nodes (Tum + LN) and tumor negative lymph nodes (tum-LN). Preliminary data during the previous year with one LN sample from one donor showed that proliferative responses to G89 by LN+ cells decrease from the distal to the diseased LN. We continued these experiments with 5 additional pairs of lymph nodes and the results were confirmed. These results are summarized in **Table III**. Significant proliferative responses were obtained from tumor-LN, but only from 2/4 tum+LN. To address the question whether the responding T cells from non proliferative tum+LN are anergic, according to the general definition of this term, cells from a pair of LN. (Tum$^+$ and Tum$^-$) were first cultured in IL-2, without peptide stimulation, then stimulated with G89, followed by culture in IL-2. Equal number of cells from the primary stimulation groups, control (NP), G89, and F13, were restimulated with NP, G89 and F13. The results and the experimental settings are shown in **Fig. 7**. These results show that G89 primed cells from Tum$^-$ cells proliferated better than cells stimulated with control (NP) or with the control peptide F13. In contrast, the levels of G89 induced proliferation were similar with the control (NP) stimulated

cells in LN$^+$ donor. These results indicate that the proliferative responses to Ag of G89 reactive cells are inhibited in LN containing tumors but this inhibition it is not a form of classical anergy, because it is not reverted by culture in IL-2, before and after Ag stimulation.

To address the fine epitope specificity of these cells they were stimulated in parallel in the same experiment with F7, G89, and F6. As reported before, F6 is a longer epitope from the same area which includes both the F7 and G89 sequences. The results are shown in **Fig. 8**. In parallel we determined the G89 concentration dependent responses of cells from Tum$^-$ LN and Tum$^+$ LN to G89. The results in **Fig. 8A** show that T cells from Tum$^-$ LN responded better to G89 than Tum$^+$ LN, suggesting that either the affinity or the frequency of G89-reactive cells is higher in Tum$^-$ than in Tum$^+$ LN. G89 induced proliferation was always higher in response to G89 than to F7 and F6. (**Fig. 8B**). This indicate that the fine specificity of LN cells is closer to G89 than to F6 and F7.

Mechanisms of G89 induced T cell help.

To better understand the effects of G89 in the stimulation of T cells by both the helper and CTL epitope we used the same DR4$^+$ donor and we stimulated its PBMC with E75 over a range of concentrations in the presence of a constant concentration of G89. The G89 induced CD4$^+$ line (G89L) obtained after two stimulations with G89 (1) was used as positive control. The results in **Fig. 9** show that in the presence of 20 μg G89 the amount of IFN-γ secreted increased dramatically and at 1 μg/ml E75 was higher than at 20 μg/ml in the absence of G89. Of interest the amount of IL-2 present slightly decreased. This suggested that the IL-2 is consumed faster than is produced. Even when 100 μg/ml E75 was added and G89 cells were present, they produced low amounts of IL-2.

Recent studies using various experimental models have confirmed the requirement for help for induction of CTL. *However, the general perception was that the CD4$^+$ help consists mainly in secretion of cytokines such as IL-2 and IFN-γ/IL-4 which modulate the CD8$^+$ cells reponse. This raise the question that if these cytokines can be provided (exogenously) why the CD4$^+$ cells are needed. Furthermore, if CD4$^+$ cells will interact with APC and lead to induction of IL-12 from APC (DC, MO), why this IL-12 is needed. Addition of exogenous IL-12, or stimulation with trimeric CD40L should be sufficient to induce IL-12.*

To address these questions we needed to set up a parallel system of stimulation: E75-DC-CD8$^+$ from the same donors. We quantitated the amounts of IFN-γ, IL-12, IL-2, (and in parallel IL-10, IL-4 and IP-10) induced at priming by E75. Our findings are summarized below and in the attached manuscript (submitted). (Lee, T.V. et al)

We investigated the ability of HER-2 peptide E75, to activate effector functions in freshly isolated CD8$^+$ cells. IFN-γ was rapidly induced by E75 within 20-24h in six of six healthy donors, in the presence of IL-12 and was detectable as early as 6h. The IFN-γ levels were Ag-concentration dependent. Similar results were obtained with peptides mapping CTL epitopes from two other tumor Ag: folate binding protein (FBP) and amino-enhancer of split of Notch (AES). IFN-γ was also detected, in response to HLA-A2 matched tumors+IL-12 but not of IL-12 alone. The major source of IFN-γ were CD45RO$^+$ CD8$^+$ cells. Induction of IFN-γ and IL-2 from CD8$^+$ cells and of IL-12 from dendritic cells (DC) by CD8$^+$ cells reactive with E75 mirrored their induction by the influenza matrix peptide (M1: 58-66) in the same individual. Responses to M1 are used to define the presence of activated memory cells in healthy individuals. Compared to M1 responses E75 recognition induced 2-4 fold lower levels of IL-12 from the same APC and IFN-γ and IL-2 from CD8$^+$ cells. At lower Ag concentrations the endogenous IL-12 induced by E75-reactive CD8$^+$ cells did not reach the threshold required to co-stimulate for IFN-γ. αB7.1 synergized with E75 in increasing the overall levels of IL-2 induced within 24h. Priming of CD8$^+$ cells with E75+IL-2+ CTLA-4/B7.1 promoted marginal proliferation suggesting a functional dichotomy in the activating effects of E75.

The conclusion of these studies is that activated CD8$^+$ cells (of memory phenotype) when present in healthy donors can induce type 1 cytokines, but their simulatory ability is just below the threshold for activation of type 1 cytokine effector function. To reach this threshold we need to use very high concentrations of CD8$^+$ epitopes (50-100 μM, = .05-.1 μg/ml).

Alternatively in the absence of Ag we need to use exogenous IL-12, CD40L or LPS-type bacterial products. However, activation of IFN-γ induction by T cells require Signal 1 = Ag + Signal 2 = IL-2 (costimulation). Thus in the absence of the Ag, IL-2, CD40L will either activate non-specific effectors, or they will activate a whole range of CTL with various unrelated specificities. The outcome of escalating IL-12 should be suppression. In contrast, the presence

of the helper Ag (such as G89 epitope) and its recognition by CD4+ cells should induce IL-12 from DC only at interaction with APC, and consequently lower the threshold for activation of CTL. by inducing responses when the Ag concentration is within feasible range (5-20 μM).

To address the question whether G89 synergy with E75 in IFN-γ induction is because of the induction of IL-12, DC were pulsed in the NP, E75, G89 and E75+G89 at concentrations (50 μg/ml) where they both can induce IFN-γ.) The results are shown in **Fig. 10**. Recognition of G89 lead to IL-12 induction from DC although at lower levels than from recognition of E75 alone. E75 + G89 had a nodest synergistic efect in enhancing the overall IL-12 levels. However, this modest synergistic effect lead to a strong synergistic effect in IFN-γ induction from the E75+G89 group. Consistent with the hypothesis that IFN-γ is induced by Ag and IL-12 has a costimulatory role. Since exogenous IL-12 was not added these results show that simultaneous stimulation with E75+G89 enhance IFN-γ levels through induction of higher levels of IL-12.

Thus, these studies identified an effect of CD4^{+} cell recognition of HER-2 helper peptide which is associated with help for CD8^{+} cells activation. This effect is due to the ability of CD4^{+} cells to activate DC to produce IL-12. The IL-12 induced by CD4^{+} cells to activate DC to produce IL-12. The IL-12 induced by CD4^{+} cells compensate for the required amount of IL-12 by CD8^{+} cells to induce IFN-γ. This IL-12 addresses the requirement for exogenous IL-12 in CD8^{+} cells activation. Our results also show that there is an additional helper effect mediated by CD4^{+} cells which is independent of IL-12. This is evident where exogenous IL-12 is provided and appears not to be dependent on IL-2 since exogenous IL-2 is also provided. Since this effect is evident in induction of cytolyte function, our studies will continue as planned to determine whether this is related to enhanced proliferation, protection from activation induced cell death of CD8^{+} cells, by interaction with CD4^{+} cells.

Furthermore, we will focus on tolerance (anergy) reversal in the lymph nodes, as planned, to address whether reversal of cytokine mediated immunosuppressive restores the ability of CD4^{+} cells to expand and they can help in induction of tumor specific CTL.

Table I. CD4 antigen expression on T cell lines from DR4$^+$ donor induced by stimulation with HER-2 peptides.

	% Positive Cells	
	CD4	**CD8**
G90*	87	11
G88	77	15
F7	76	18
F13	70	15
F14	91	9
TT (Control)*	93	4

* Indicates significant increase in CD4 Ag expression compared to the other lines, TT (tetanous toxoid) was used as positive control.

Table II. Requirements for help for induction of E75 specific CTL.

DC	E75	CD4$^+$ Line	F7	IL-12	% Specific Lysis	
					E75	**D132**
+	+	+	-	-	2.5	0.0
+	+	+	+	-	13.5	0.0
+	+	+	-	+	12.1	0.0

Dendritic Cells (DC) were cultured in GM-CSF + IL-4. Responder T-cells consisted of the plastic non-adherent fraction of HLA-A2$^+$ donor's PBMC. The CD4$^+$ line was maintained by stimulation with HER-2 peptide F7. D132 is an unnatural mutated MUC-1 peptide: D132, S L A D P A H G V. CTL activity was determined at an E:T ratio of 10:1. IL-12 was used at 300 pg/ml. Targets were T2 cells pulsed with E75.

Table III. Decreased proliferative response of T cells from Tum$^+$ lymph nodes to HER-2 peptide G89.

	Proliferation *	
Sample	Tum$^-$	Tum$^+$
LN1	+	-
LN2	+	+
LN3	+	N.D **
LN4	+	-
LN5	+	+
Total responding:	**+5/5**	**2/4**

* A response was considered positive when the stimulation index was > 1.6 and the difference between replicates were considered significant ($p < 0.05$).

** The amount of lymphocyte material was not sufficient for proliferation assays.

Legends to the figures.

Figure 1. Surface antigen expression on dendritic cells from a DR4$^+$ donor after stimulation with G89 peptide alone (■). or peptide plus isolated autologous T cells (■). Note the marked upregulation of HLA-DR (4.5 fold), MHc-I (3.5) followed by CD40, but <u>not</u> of B7.1. Results are expressed as MCF (mean channel fluorescence). Surface antigen expression was determined 24h after incubation with T cells and peptides.

Figure 2. Recognition of G89 on DC by G89 stimulated cells (g89P) upregulates CD40 Ag expression . E75 and G89 synergize in upregulation of IFN-γ induction. G89P, and E75P indicate that T cells from DR4$^+$ donor were primed (P) with G89 and E75 respectively. Supernatants were collected and the levels of IFN-γ, (A) and of CD40 expression (B) were determined 20h later.

Figure 3. Conditioning of APC (DC) by the interaction between G89-primed cells and G89 presented on APC is required for induction of CTL activity recognizing HER-2 peptide E75. DC were pulsed either with no peptide (DC). or with E75 (DC-E75) or both G89 (DC-G89). DC and DC-E75 were then incubated with E75 primed cells. DC-G89 were incubated either with G89 primed cells alone, or with DC-G89+E75+E75 primed cells. G89 and E75 primed cells were stimulated only once with peptide. CTL activity was determined 5 days later, using as targets T2 pulsed with no peptide (T2-NP) or T2 pulsed with E75 (T2-E75). Induction of specific CTL activity at restimulation was detected only in the DC-G89, - E75, +G89P + E75P group.

Figure 4. Increased proliferation of DR4$^+$ T cells at restimulation with the combination of CTL epitope (E75 = E) plus the helper epitope (G89 = G). Equal number of cells of the DR4$^+$ (E+G) T cell line were stimulated with E75 (●), G89 (■), E+G (E75 + G89) (**O**), or as control E75+D100 (□). D100 is another HER-2 peptide which lacks stimulatory ability. Autologous DC were used as APC. IL-2 was added in the cultures 48h after Ag stimulation. The proliferation index was determined by dividing the number of recovered live cells after 8 days of stimulation with the number of live cells at the initiation of stimulation.

Figure 5. Stimulation of HLA-A2$^+$ tumor infiltrating lymphocytes from a breast cancer patient, with the CTL epitope E75 together with the helper epitope F13 augments recognition of E75

pulsed target T2 cells by CTL-TIL. E:T was 10:1. The effects are F13 specific since F7 does not induce an increase in specific recognition. Results of a 5 h CTL assay.

Figure 6. Priming and restimulation of T cells from an HLA-A2$^+$ HLA-DR4$^+$ donor with E75 + G89 leads to induction of specific CTL activity detected in a 5 h CTL assay. E:T ratio was 8:1. (**) = indicate significant differences (p < 0.05), NP and E75 indicate that 51 Cr-labelled T2 cells were pulsed either with medium control (no peptide) or with E75. Details on stimulation groups in pages 11-12.

Figure 7. Proliferation of G89 reactive cells is impaired in breast tumor <u>positive</u> lymph nodes, but not in breast tumor <u>negative</u> lymph nodes. Culture in IL-2 and restimulation of LN cells from the tumor positive LN with G89 does not enhance their proliferation suggesting that the responders are anergized. This possibly involves different mechanisms than classic anergy.

Figure 8A. Concentration dependent recognition of G89 by LN cells from Tum$^-$ and Tum$^+$ LN. from a cancer patient **8B**. F6 and F7 are recognized less than G89 by the same cells from Tum$^-$ and Tum$^+$ LN. F6 and F7 were used at 25 μg/ml.

Figure 9. HER-2 helper peptide G89 enhanced E75 induced IFN-gamma secretion. IFN-γ in supernatants collected 24 hours after stimulation was measured with IFN-γ specific ELISA. 2xG89 indicate that autologous T cells stimulated twice with G89 (G89L) were added.

Figure 10. The synergy E75 + G89 in IFN-γ induction correlates with an additive effect of E75 + G89 in IL-12 induction. DC were pulsed with NP, E75, G89, and E75 + G89. Autologous plastic nonadherent PBMC were used as responders. Supernatants were collected 20 h later and used for determination of IL-10, IFN-γ and IL-12 in the same experiment.

Conclusions

(1) We found that interaction of isolated HLA-DR$^+$ cells with autologous DC cells (APC) lead to upregulation of HLA-DR, HLA-ABC, and CD40, but a lesser extent of B7.1. This effect was mediated by specific recognition of G89. since interaction of G89 cells alone with APC did not lead to upregulation of CD40.

(2) We generated cell lines from a HLA-DR4$^+$ donor by stimulation with HER-2 peptides. These lines are predominantly 75-90% CD4$^+$. Interaction of these lines with APC (DC) presenting the helper epitopes F7 + F13 helps expression of cytolytic function by CTL stimulated with the CTL epitope peptide E75. A similar effect was observed when TAL from a patient with breast cancer were stimulated with E75 + F13/F7. Thus, indeed these helper epitopes help in expression of CTL function.

(3) Simultaneous stimulation with the CTL + helper epitope is necessary and required for HER-2 specific CTL induction. This was confirmed in two independent donor systems. Specific CTL activity was detected only in cultures initiated with E75 + G89. The reason for this requirement appeared to be related to the fact that APC were required to present G89 and to interact with G89 primer cells.

(4) We found that recognition of G89 leads to activation of DC and induction of IL-12, but not of IL-10. This indicate that activated (expressing CD40L) CD4$^+$ cells exist in the healthy donors and patients. Similarly we found that activated CD8$^+$ memory cells exist in these individuals (in a parallel study. Recognition by CD8$^+$ cells of the tumor Ag induces IL-12 but the levels of IL-12 are just below the levels required to costimulate with Ag IFN-γ induction. Thus the additional levels of IL-12 induced by CD4$^+$ cells, recognized G89, overcame the need for exogenous IL-12 for activation of type 1 response.

(5) We extended and confirmed our previous findings on G89 recognition by lymph node cells. G89-reactive cells are present predominantly in the lymph nodes without disease. In the diseased lymph nodes, this response is suppressed. The mechanism responsible for this suppression likely involve clonal anergy, since the G89 primed cells from tumor + LN, even after culture in IL-2 do not respond at restimulation with G89.

In summary, studies done this year demonstrated that the helper epitope G89 (or HER-2 epitopes with CD4$^+$ stimulating function) are required for priming and induction of a CTL response to the immunodominant HER-2 epitope E75. We plan to reverse the tolerance observed in the lymph nodes by conditioning of DC for inflamatory responses, and the use of antibodies to TH2 cytokines, and design of G89 analogs which may be more potent activators of a helper response for CTL induction.

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APPENDICES

Publications Resulting from this Grant

Lee, T.V., Anderson, B.W., Castilleja, A., Peoples, G. E., Wharton, J. T., Murray, J. L., Ioannides, C. G. Identification of activated tumor antigen-reactive $CD8^+$ cells in healthy individuals. (Submitted)
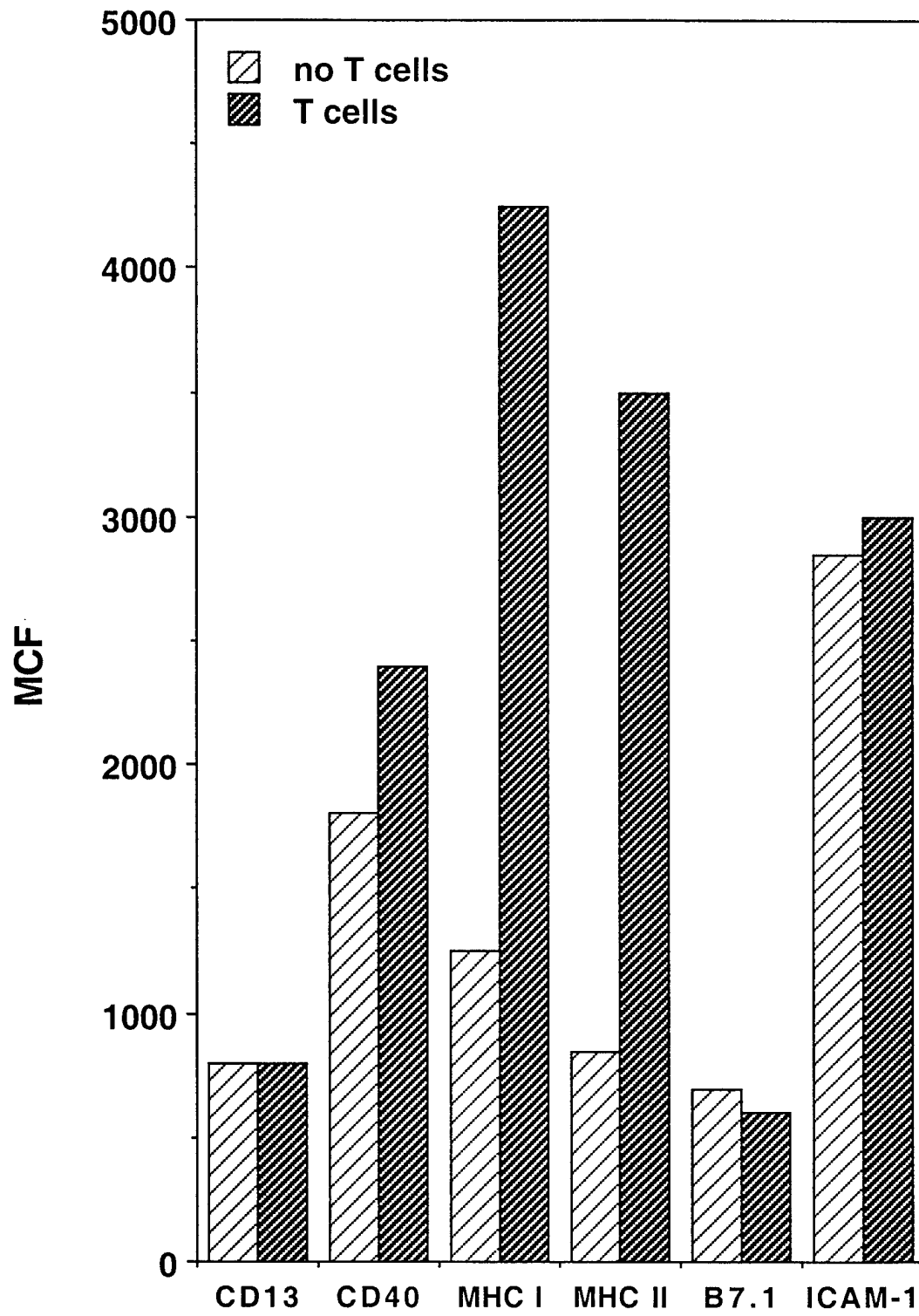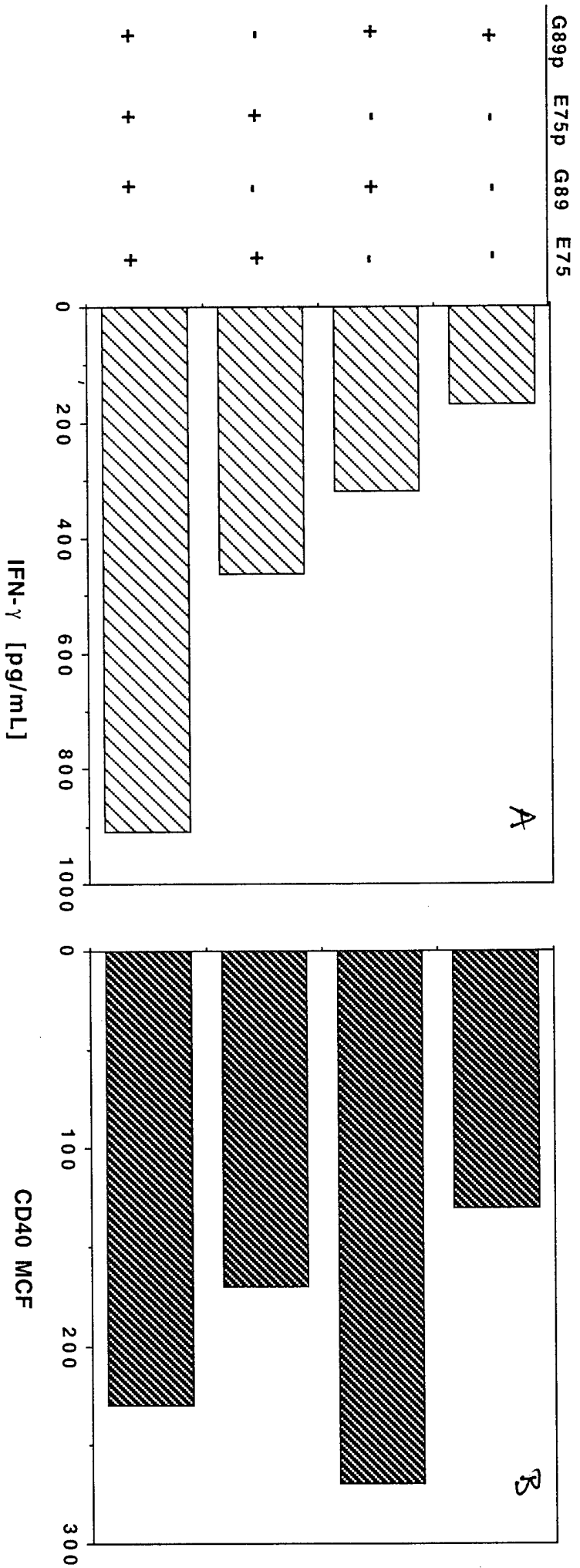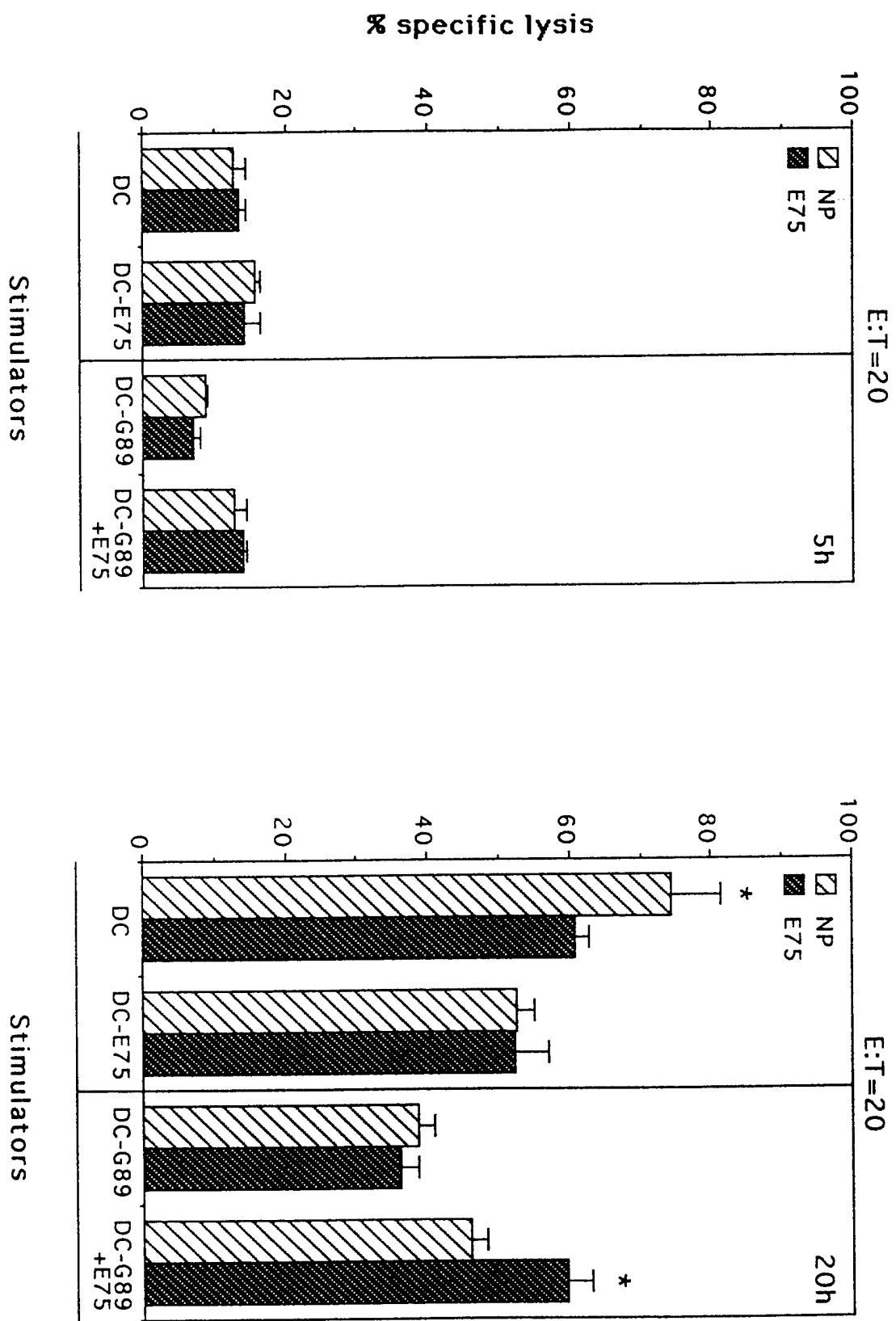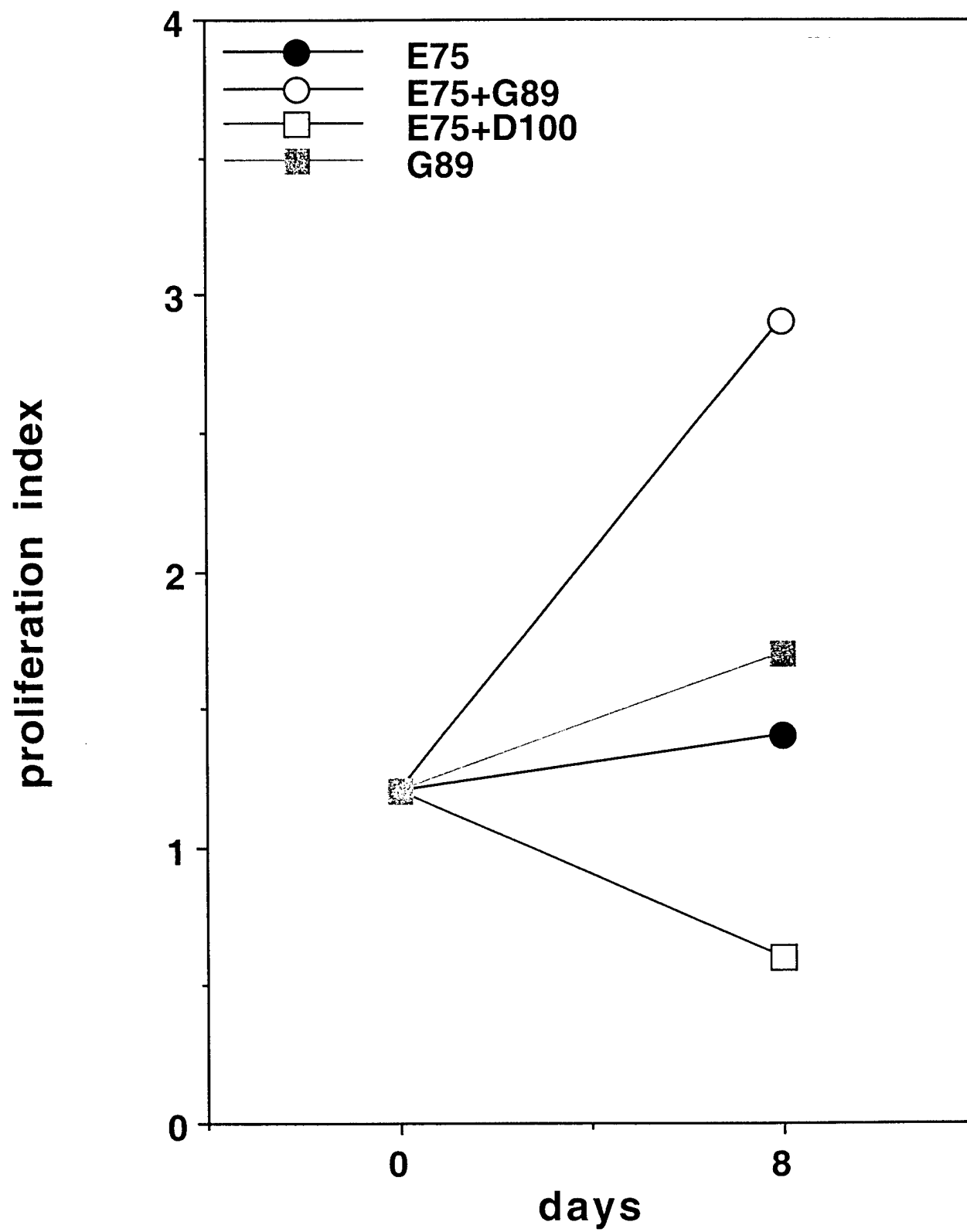
Figure 1

Figure 2

G89p E75p G89 E75

+ + + +

- + - +

+ - + -

+ - - -

IFN-γ [pg/mL]

0   200   400   600   800   1000

A

CD40 MCF

0   100   200   300

B
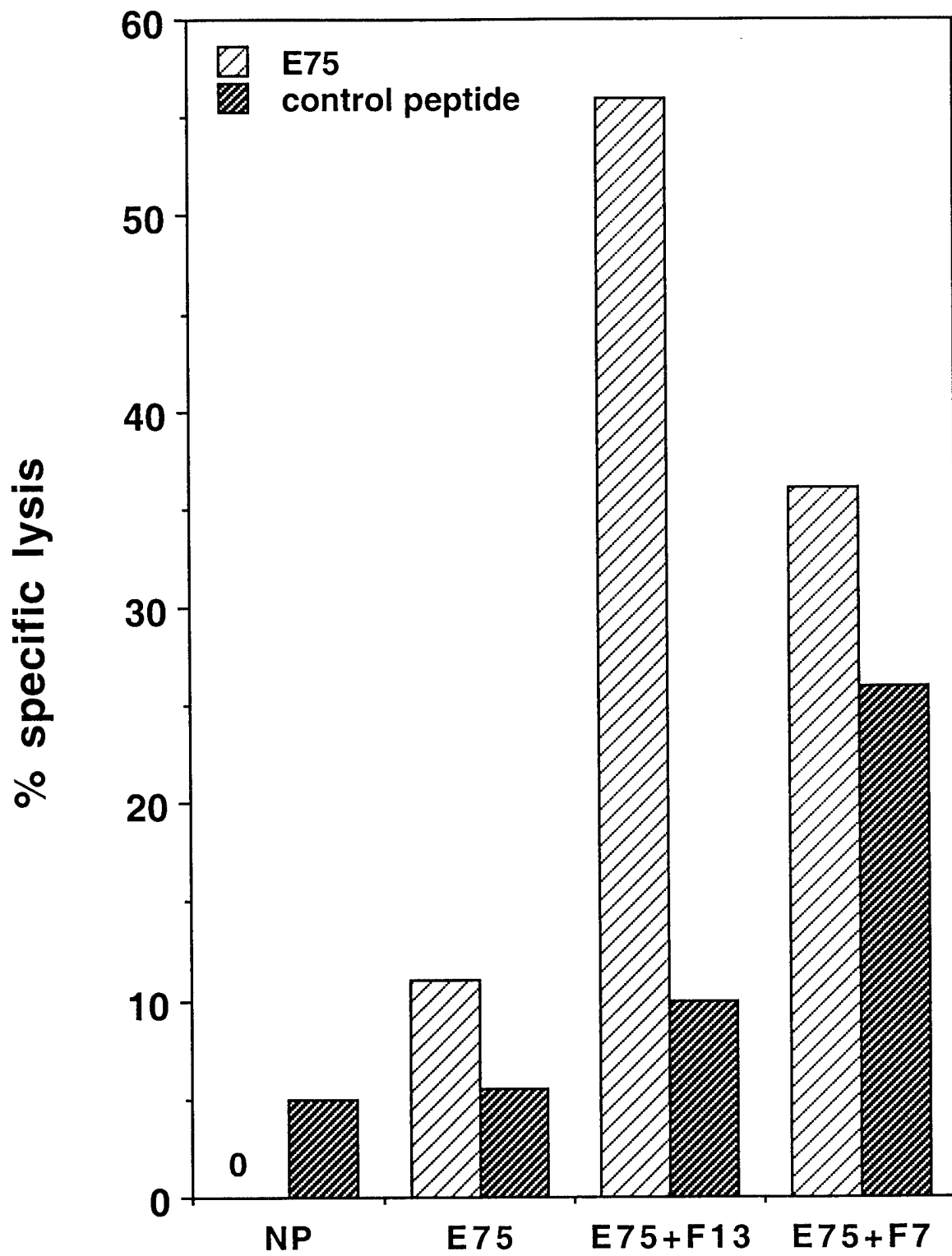
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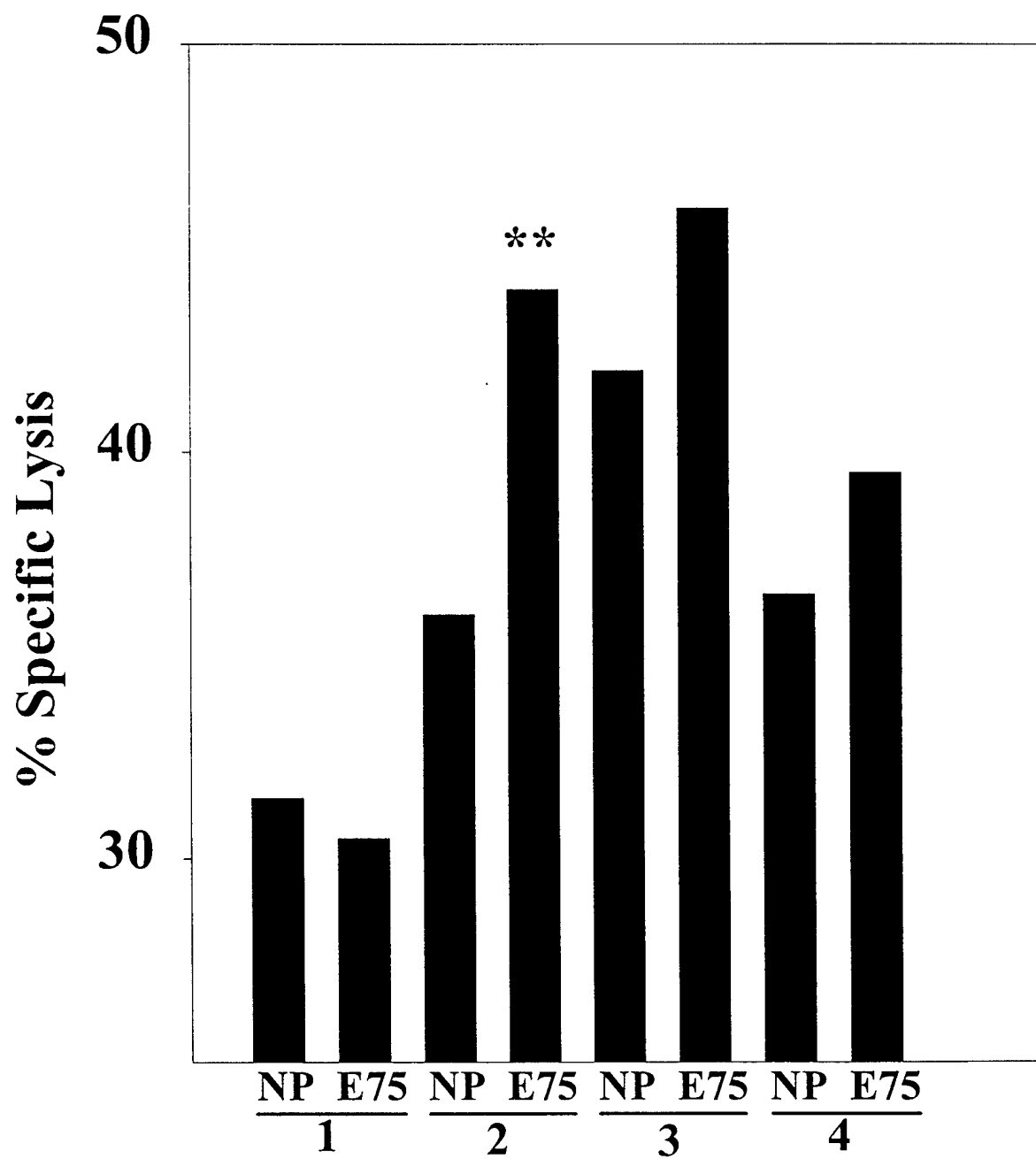
Figure 4

Figure 5
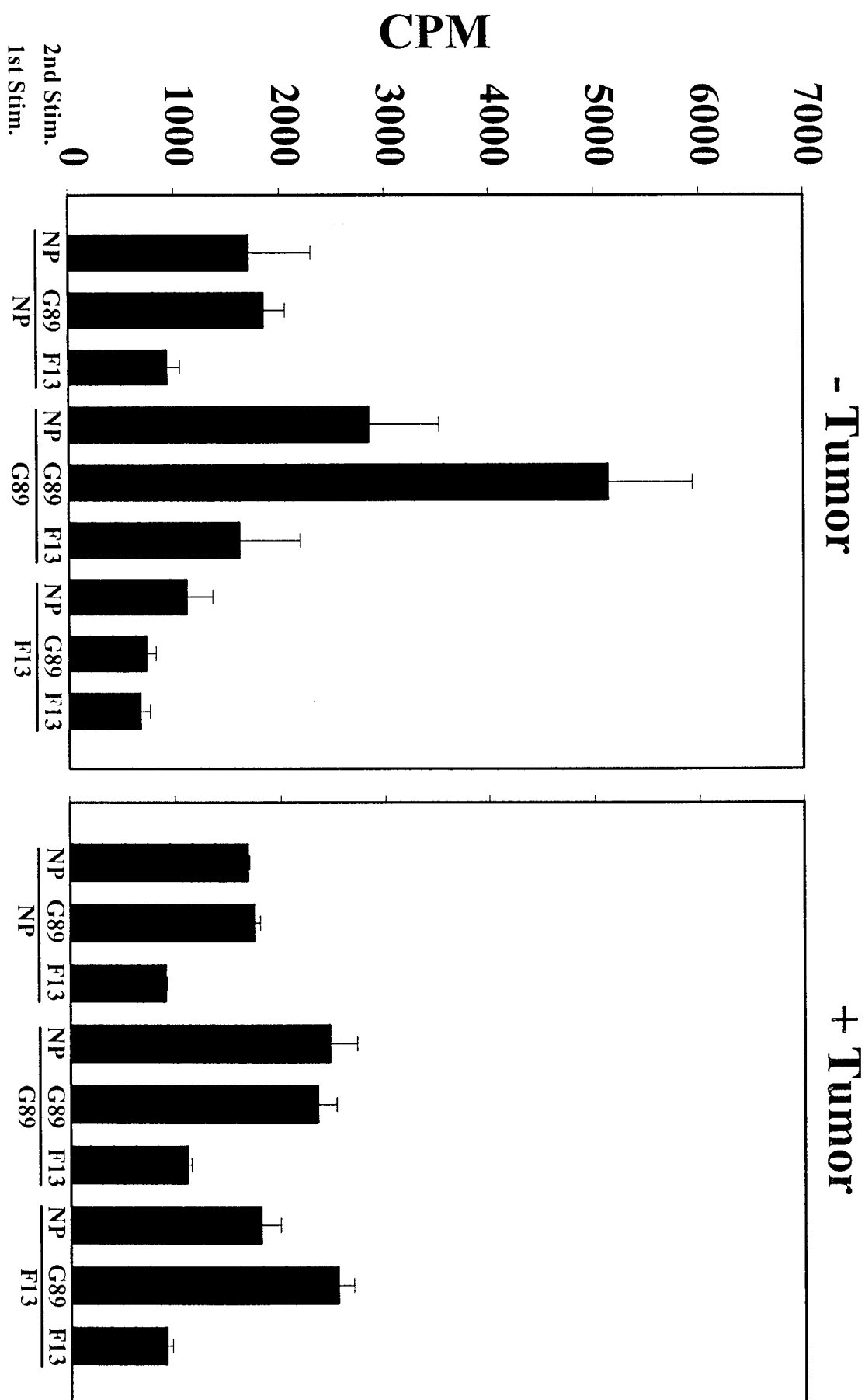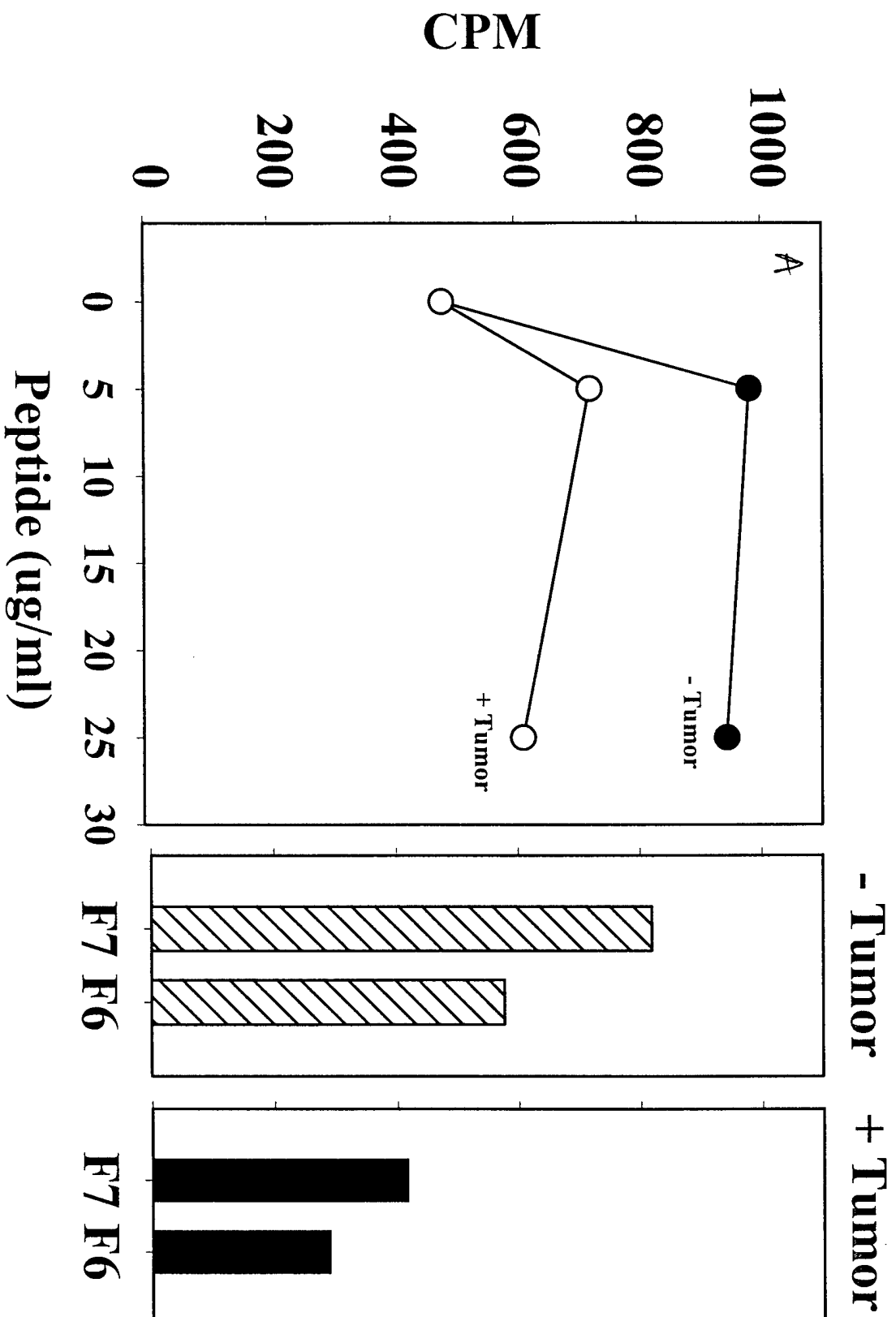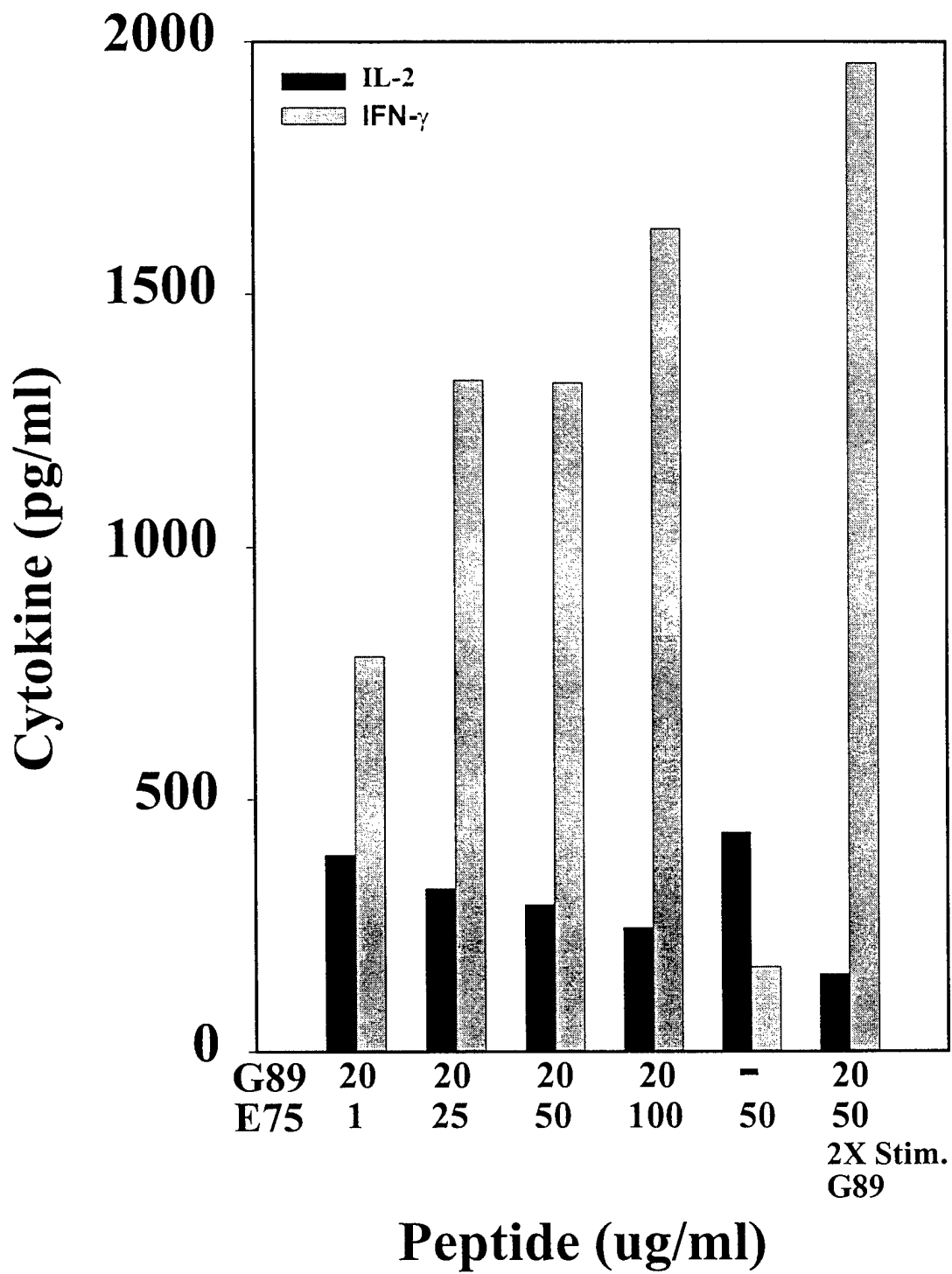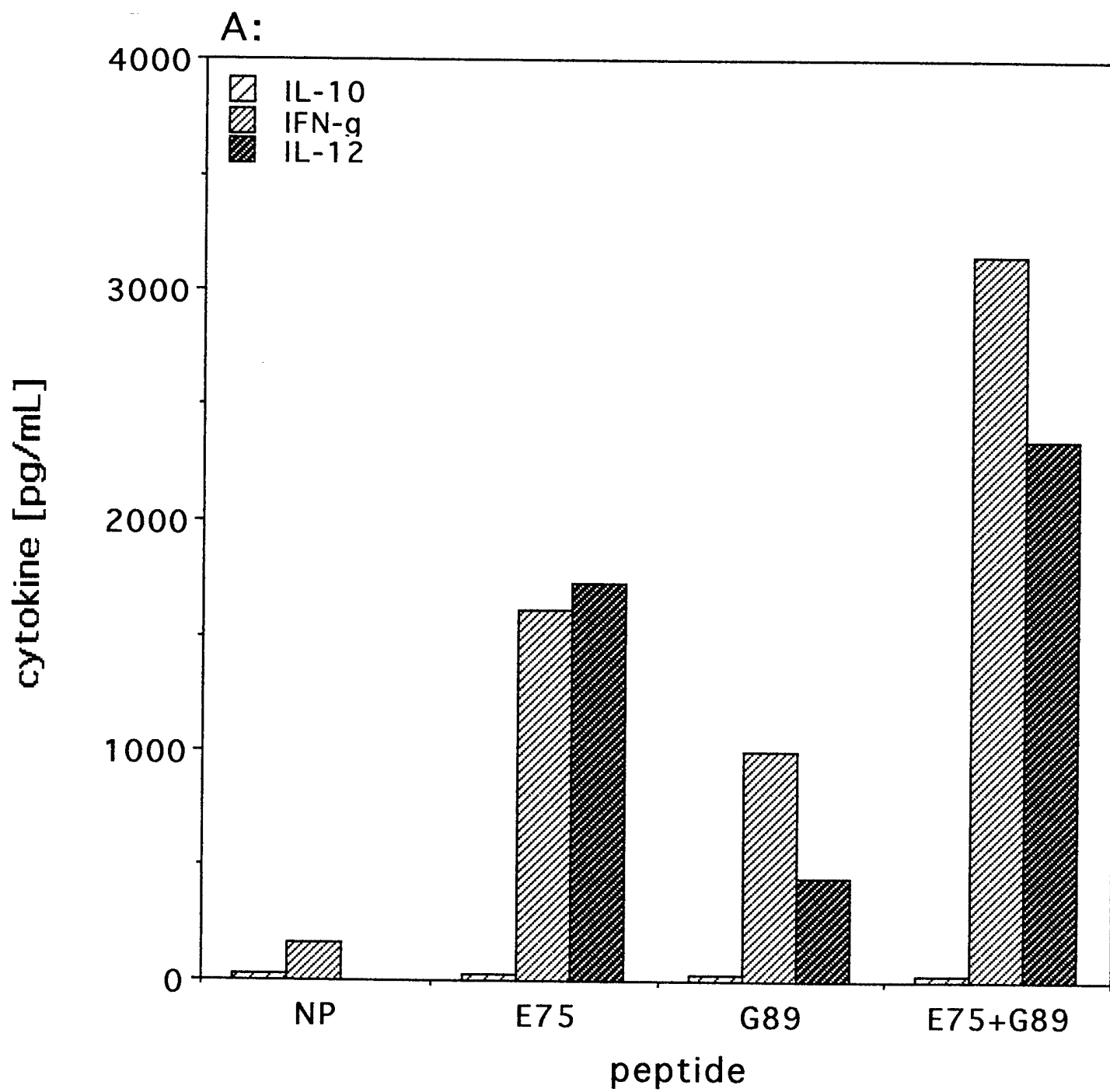
Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

A:

Identification of activated tumor antigen-reactive CD8^{+} cells in healthy individuals.

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Running Title: Activated HER-2 reactive memory CD8

Key words: CTL, HER-2, interferon-γ, IL-12, IL-2, CTLA-4, B7

[1]*Abbreviations used in this paper*: $\propto$, anti; DC, dendritic cells; HER-2, HER-2/neu protooncogene, TAL, tumor associated lymphocytes, HS, human serum; NP, no peptide; AES, aminoenhancer of split; FBP, folate binding protein.

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Summary

We investigated the ability of HER-2 peptide E75, which maps an immunodominant CTL epitope for ovarian and breast tumor-associated lymphocytes (TAL)[1], to activate effector functions in freshly isolated CD8$^+$ cells. IFN-γ was rapidly induced by E75 within 20-24h in six of six healthy donors, in the presence of IL-12 and was detectable as early as 6h. The IFN-γ levels were Ag-concentration dependent. Similar results were obtained with peptides mapping CTL epitopes from two other tumor Ag: folate binding protein (FBP) and amino-enhancer of split of Notch (AES). IFN-γ was also detected, in response to HLA-A2 matched tumors+IL-12 but not of IL-12 alone. The major source of IFN-γ were CD45RO$^+$ CD8$^+$ cells. Induction of IFN-γ and IL-2 from CD8$^+$ cells and of IL-12 from dendritic cells (DC) by CD8$^+$ cells reactive with E75 mirrored their induction by the influenza matrix peptide (M1: 58-66) in the same individual. Responses to M1 are used to define the presence of activated memory cells in healthy individuals. Compared to M1 responses E75 recognition induced 2-4 fold lower levels of IL-12 from the same APC and IFN-γ and IL-2 from CD8$^+$ cells. At lower Ag concentrations the endogenous IL-12 induced by E75-reactive CD8$^+$ cells did not reach the threshold required to co-stimulate for IFN-γ. αB7.1 synergized with E75 in increasing the overall levels of IL-2 induced within 24h. Priming of CD8$^+$ cells with E75+IL-2+ CTLA-4/B7.1 promoted marginal proliferation suggesting a functional dichotomy in the activating effects of E75. The presence of tumor Ag-reactive activated CD8$^+$ cells in unimmunized healthy individuals may improve our understanding of the mechanisms of immunosurveillance and regulation of immune responses by tumors.

Introduction

The recent characterization of tumor Ag recognized by CTL opened the possibility of development of Ag and epitope-specific cancer vaccines. Tumor Ag recognized by melanoma, ovarian, and breast CTL have been demonstrated to be self-proteins (1). The fact that in cancer patients, CTL-specific for these self peptides co-exist with progressive tumors, suggest that such responses can be primed in vivo, but either CTL do not expand to the numbers required to mediate an effective response, or they expand but they are not functional in their state in vivo. This raises the question whether: (a) such CD8$^+$ cells are already present in healthy individuals; (b) represent naive (ignorant), activated or activated and tolerized effectors; and (c) if they are ignorant and tolerized which mechanisms are involved. Since tumor derived factors such as IL-10 inhibit priming of type 1 responses to tumor Ag (2) clarification of the presence and requirements for activation of these cells may be beneficial for preventive cancer vaccination in high risk individuals or patients with no evidence of disease after chemotherapy.

If the Ag targets naïve cells, they will respond if costimulatory receptors are present on APC and bind to their appropriate ligands. An effector response by cytokines will be observed after progression through the cell cycle and 2-4 divisions (i.e. 30 - 40 h). (3) This response will be inhibited by antibodies to costimulatory molecules such as B7.1/B7.2. "Ignorant" cells may require increased TCR signaling which can be achieved by Ag modification (4). However, the kinetics of IFN-γ secretion and requirement for B7-CD28 will not change. If Ag induces tolerance, then naive cells may express a partial response at priming but they will not develop a response at restimulation (5). If activated effectors are present and are tolerized/anergized by exposure to Ag in the absence of costimulation, they will be unable to respond to the cognate/crossreactive stimulus that was initially effective for their activation. Analysis of the cytokine response can distinguish whether Ag induces clonal anergy (characterized by minimal secretion) or cytokine mediated immunosuppression due to high levels of IL-10 (6). In contrast, if activated effectors are present, they will immediately or rapidly respond to Ag by cytokine secretion without requiring division (7). In this case costimulation through surface receptors may have an enhancing/stabilizing effect on some responses (e.g. CD28 on IL-12 R) and a regulatory effect on other responses due to B7 ligation

by negative signaling receptors (e.g. CTLA-4) present on activated cells (8). Thus characterization of the patterns of reactivity of PBMC to tumor Ag can provide an answer to these questions.

To address the presence of activated effectors in the absence of disease, and establish the ability and limitations of tumor Ag to activate CD8$^+$ cells effector functions, we investigated the ability of peptide E75, HER-2 (369-377) (9), to stimulate freshly isolated, peripheral blood CD8$^+$ cells from healthy unimmunized individuals, when pulsed on CD13$^+$ CD14$^-$ DC (DC-E75). E75 is not only recognized by *in vivo* generated ovarian CTL-TAL, but in vitro restimulated ex vivo primed CTL-TAL from cancer patients to mediate specific tumor lysis (10, 11). The advantage of this model system is that the effects of defined tumor Ag on activation of peripheral T cells from healthy donors are not perturbed, or polarized by prior in vitro culture with Ag+cytokines. This also adds a component of physiologic relevance to the activation pathways investigated since Ag+cytokine induced activation may be critical to the patient's response to tumors.

Since the frequency of CD8$^+$ cells bearing TCR capable of recognizing tumor Ag may be low and primary stimulation of PBMC with most tumor Ag, including HER-2 is inefficient in induction of detectable specific cytolytic effectors we focussed our analysis on cytokine induction by peptides corresponding to CTL epitopes from HER-2 (9-12) and the newly identified tumor Ag: folate-binding protein (FBP) and amino-enhancer of split (AES) (13, 14). Analysis of cytokine induction in T cells can detect response patterns to Ag, the presence of activated memory effectors (6), the ability of the tumor peptide to induce a type 1 (inflamatory response), and the requirements for co-stimulation for amplification of this response. Second, HER-2^{hi} tumors are sensitive to IFN-γ. IFN-γ inhibits their proliferation, HER-2 overexpression, enhance MHC-I expression in tumor cells, rendering them more sensitive to CTL mediated lysis and induce tumors to secrete factors with angiostatic activity (e.g. IP-10) (15, 16).

DC-E75 stimulated high and rapid Ag specific IFN-γ, but not IL-4 and IL-10 secretion by these CD8$^+$ cells in all donors in the presence of IL-12. In the absence of IL-12, rapid induction of IFN-γ required higher concentrations of E75. DC-E75 also induced IL-2 in these cells. αB7.1 enhanced several-fold IL-2 induction by E75. E75 induced IFN-γ from CD8$^+$ CD45R0$^+$ cells suggesting that the responders were activated memory cells. IFN-γ production was under the

control of the levels of endogenous IL-12 secreted by DC-E75 interacting with freshly isolated CD8$^+$ cells. Compared with a conventional Ag, the influenza matrix peptide M1, which is used to define the presence of activated memory effectors in healthy individuals, the levels of cytokines induced by E75 were several fold lower, but they were similar when 4 fold more E75 was used as inducer. The primary DC-E75 stimulation even in the presence of $\propto$B7.1, or $\propto$–CTLA-4 enhanced weakly T cell proliferation and did not enhance specific cytotoxicity, although it enhanced IL-2 production. Our results indicate that activated memory cells reactive with E75 and other tumor peptides are frequently present in healthy donors. They may be activated by self-Ag and in *trans* by cytokine secreted in response to environmental pathogens. They appear to be neither Ag ignorant nor functionally anergized, but the tumor Ag acts as a weak/partial agonist by selectively inducing only a subset of CD8$^+$ effector functions.

Materials and Methods

Cells. HLA-A2$^+$ PBMC were obtained from healthy volunteers from the Blood Bank of M.D. Anderson Cancer Center. The HLA phenotypes of the donors used in this study are as follows: Donor 1 (A2, B7, 44), Donor 2 (A2, 33, B40, 44), Donor 3 (A2, 33, B41, 81), Donor 4 (A1, 2, B27, 44), Donor 5 [A1, 2 B44, 57, Cw5, 6), Donor 6 (A2, 31, B35, 44, Cw4, w5). T2 cells, ovarian SKOV3, SKOV3.A2 cells, and indicator tumors from ovarian ascites were described (9).

Antibodies and Cytokines. mAb to CD3, CD4, CD8 (Ortho), CD13 and CD14 (Caltag Laboratories, San Francisco, CA), B7.1 and B7.2 (CD80 and CD86, Calbiochem), ICAM-1 (CD54, Calbiochem), CD40L (Ancell, Bayport, MN), HLA-A2 (clone BB7.2, ATCC), and MHC-II (L243, DAKO Corp., Carpintera, CA) were used as unconjugated FITC or PE conjugated. Anti-CTLA-4 was a kind gift from Dr. Peter Linsley (Bristol-Myers). mAb specific for IL-12, IFN-γ and isotype controls were obtained from Pharmingen. The following cytokines were used: GM-CSF (Immunex Corp., Washington, DC), specific activity 1.25 x 10^7 CFU/250 mg; TNF-α (Cetus Corp., Emeryville, CA), specific activity 2.5 x 10^7 U/mg IL-4 (Biosource International), specific activity, 2 x 10^6 U/mg; IL-2 (Cetus), specific activity 18 x 10^6 IU/mg; IL-12 of specific activity 5 x 10^6 U/mg was a kind gift from Dr. Stanley Wolf, Department of Immunology, Genetics Institute, Cambridge, MA.

Synthetic peptides. The HER-2 peptides used were: E75 (369-377) GP2/F53: (IISAVVGIL, 654-662), and F57(IHLNGSAYSL, 439-447). GP2 and F57 define HER-2 CTL epitopes distinct from E75 (11-12). The modified Muc-1 peptides used were D125: (GVTSAKDTRV) and D132 (SLADPAHGV). The FBP peptides used were: E39(FBP,191-199, EIWTHSYKV), and E41(FBP, 245-253 LLSLALML). The Amino Enhancer of Split (AES) peptide used was G76:GPLTPLPV. FBP and AES peptides were recently identified to be recognized by ovarian and breast CTL (13, 14). The positive control CTL epitope used was the influenza matrix peptide (58-66): GILGFVFTL, designated as M1. M1 forms an immunodominant epitope recognized by memory CTL in healthy donors (7). All peptides were prepared by the Synthetic Antigen Laboratory of M.D.

Anderson Cancer Center and purified by HPLC. Peptides were 95-97% pure by amino acid analysis. Peptides were dissolved in PBS and stored frozen at –20 °C in aliquots of 2 mg/ml.

Immunofluorescence. Antigen expression by DC and T cells was determined by FACS using a flow-cytometer (EPICS - Profile Analyzer, Coulter Co, Hialeh FL). DC were defined by the presence of CD13 and absence of CD14 marker after culture in GM-CSF and IL-4. For phenotype analysis, DC were incubated with PE-conjugated anti CD13 mAb and FITC-conjugated mAb specific for a surface Ag. For determination of the effects of cytokines, peptides and T cells on surface antigen expression, DC were incubated with the same amounts of cytokines and peptides as in T cell activation assays for 24 h, and the levels of Ag expression were determined in the gated $CD13^+$ population.

Culture of PBMC-derived DC. $CD13^+$ DC were generated from freshly isolated PBMC following the established CD14 methods (17, 18). Complete RPMI medium (containing 10% FCS) supplemented with 1000 U/ml GM-CSF or 500 U/ml IL-4 was added to each well containing plastic-adherent cells and maintained for 7 days. T cells were obtained from the plastic non-adherent PBMC by removal of $CD16^+$ and $CD56^+$ cells. $CD8^+$ cells were isolated by removing first the $CD4^+$, and then the $CD16^+$ and $CD56^+$ cells from the nonadherent population using Dynabeads (Dynal, Oslo, Norway). $CD8^+$ subpopulations were obtained using anti-CD45RO mAb and anti-CD45RA mAb (UCHL-1, DAKO) as described (7). After depletion, the resulting cells were 97% $CD8^+$ as determined by flow cytometry.

T cell stimulation by peptide pulsed DC. We used $CD13^+$ DC, as APC because such cells have been reported to activate both naive and memory CTL (19). After culture in GM-CSF+IL-4, DC from all donors were >96% $CD13^+$, and $CD14^-$ expressed high levels of MHC-I, MHC-II, CD54, CD40, and CD86, but lower levels of CD80 in agreement with the described phenotype of peripheral blood $CD14^+$ derived DC (18). DC were plated at 1.2 x 10^5 cell/well in 24-well culture plates, and pulsed with peptides at 50 μg/ml in serum-free medium for 4 h before addition of responders. TNF-∝ (50 U/ml) was added to DC for the last hour to stimulate Ag uptake and presentation (17). Autologous, isolated $CD8^+$ cells or isolated $CD8^+$ cells ($CD45RO^+$ and CD45R0+cells depleted) in RPMI 1640 containing 10% HS were added to DC at 1.5 x 10^6/ml,

followed by IL-12. IL-2 was added 12-16 h later to each well. For inhibition studies, mAb specific for B7.1, B7.2, HLA-A2 and isotype control MOPC myeloma were added to DC or tumor cells, 1 h before responders in amounts reported to be inhibitory by the manufacturers. Anti CTLA-4 and CD40L were added to T cells 1 h before they were added to cultures. The effects of peptides and cytokines on T cell survival were determined by counting the numbers of recovered viable cells, and determinating the numbers of $CD8^+$ and $CD4^+$ cells in the sample by flow-cytometry. Specific proliferative responses to E75 were determined by measuring the incorporated radioactivity in equal cell numbers pulsed with 1 μCi of (^{3}H)-TdR (20).

CTL and cytokine assays. Recognition of peptides used as immunogens by CTL was performed as described (11). Equal numbers of viable effectors from each well were used in all assays. Supernatants collected at 6, 24, or 48 h were tested in duplicate for the presence of IL-2, IL-4, IL-10, IL-12 and IFN-γ using cytokine ELISA-kits -(Biosource International, Camarillo, CA) or R&D systems as described (20) with a sensitivity of 4-7 pg/ml. IL-12 was detected using an ELISA kit which recognizes both p40 and the natural heterodimeric molecule. The amount of cytokines was quantitated using standard plots of known concentrations of cytokines determined in the same experiment.

Results

CD8$^+$ cells from healthy donors display specific IFN-γ secretion within 24 h of contact with HER-2 peptide E75, Potentiation by IL-12. To address whether stimulation with E75 induce cytokine responses, plastic-nonadherent PBMC from healthy donors were stimulated with autologous DC pulsed with E75 (DC-E75) or as control with DC which were not pulsed with peptides (DC-NP). Supernatants were collected 20-24 h later and tested for the presence of IFN-γ. Since E75 was poorly immunogenic for activation of cytolysis in PBMC (21), and the frequency of E75-specific responders may be low, we rationalized that addition of low concentrations (100-300 pg/ml) of IL-12 will amplify the levels of IFN-γ induced by E75, thus increasing the likelihood of detection of E75-responsive T cells. IL-12 acts as co-stimulator for IFN-γ induction from T cells by Ag, but by itself is a weak inducer of IFN-γ. (22, 23) Results in **Fig. 1** show the pattern of IFN-γ responses to E75 from four healthy donors, in the absence or presence of IL-12. In Donor 1 in some experiments performed over time E75 rapidly induced IFN-γ without exogenous IL-12 (**Fig. 1A**), while in Donor 2 (**Fig. 1B**) IL-12 was required to induce detectable IFN-γ levels to E75.

Since these experiments were performed with populations and not with isolated CD8$^+$ cells, these experiments were repeated three times at weekly intervals with Donor 3 to address whether rapid IFN-γ induction and its amplification by IL-12 was not an isolated event. From Donor 3 it was possible to obtain repeatedly peripheral blood over six months. Most experiments shown here were performed with Donor 3 and were confirmed with at least one randomly selected HLA-A2$^+$ donor. Each experiment shown in **Fig. 1C** was performed with blood samples collected in different days. We used each time freshly cultured DC, plastic nonadherent PBMC from the same sample, and since the amount of blood was small, variable concentrations of E75 (20-50 µg/ml) and IL-12 (150 or 300 pg/ml). The results of stimulation experiments over a period of 3 weeks confirm that the pattern of responses observed with Donors 1 and 2 was not an isolated event. At these E75 concentrations, IFN-γ was undetectable unless amplified by IL-12. In Donor 5 E75 in high concentrations (100 µg/ml) induced high levels of IFN-γ within 24 h in the absence of exogenous IL-12 (**Fig. 1C, Column E**). Similar results were obtained with two other HLA-A2$^+$ donors (**Fig. 3B, 6A**) which were tested in separate experiments.

The IFN-γ levels continued to increase during the first 96h of E75 stimulation. IL-4 was not detected in the E75 stimulation supernatants while the levels of IL-10 determined in the same experiment did not exceed 10 pg/ml during the first 96h of E75-stimulation (data not shown). Similar results were obtained with 5 of 6 unimmmunized HLA-A2$^+$ breast cancer patients tested in the same conditions. Representative results with one patient in **Fig. 1C** also show that the IFN-γ response was also enhanced by $\propto$-CTLA4 mAb. These results suggest that there are significant numbers of E75-reactive T cells in the PBMC of unimmunized healthy individuals and cancer patients that could be readily recalled following a primary stimulation in vitro by E75 $\pm$ IL-12.

T cells from healthy donors secrete IFN-γ within 24h in response to tumor cells in the presence of IL-12. Identification of E75 reactivity with high frequency in the PBMC raise the question of the potential of these cells for tumor recognition. To address this question, we investigated whether freshly isolated Donor 1 T cells recognized better the HLA-A2 matched tumor SKOV3.A2 compared with the non-matched SKOV3. SKOV3 and SKOV3.A2 are identical but the latter express a transfected HLA-A2 gene. Thus, Donor 1 and SKOV3.A2 (HLA-A2, 3, 28, B18, 35) shared only HLA-A.2. We wanted to know whether the same or higher levels of IFN-γ will be induced in T cells responding to Ag presented by allo-MHC-I compared with common HLA-A2. The results, **Fig. 2A** show that T cells secreted low IFN-γ levels in response to SKOV3.A2. IFN-γ was not detected in response to SKOV3. In the presence of IL-12, IFN-γ was detected in both cultures within 24h, but the levels were significantly higher in response to SKOV3.A2 than to SKOV3, suggesting that HLA-A2 restricted activated T cells are present in this donor. To verify that IFN-γ production was the result of HLA-A2 recognition, SKOV3.A2 cells were incubated either with BB7.2 mAb ($\propto$ - HLA-A2) or with a non-specific isotype control Ab (MOPC). The results (**Fig. 2B**) confirmed that most of the IFN-γ was produced in response to HLA-A2, and was not increased by higher concentrations of IL-12. Thus, freshly isolated T cells from healthy donors can recognize tumors in an MHC restricted fashion without previous in vitro stimulation.

E75 induce IFN-γ secretion from activated memory cells. Requirement for cognate Ag. Rapid induction of IFN-γ by E75 raised the questions whether E75 and other tumor peptides prime naïve T cells or whether activated T cells of this specificity are present in these healthy donors. Naïve T cells and resting memory cells require cell cycling (at least 1-2 divisions) i.e., 30-40 h after

Ag stimulation to secrete IFN-γ, while activated memory effector CTL respond to Ag by IFN-γ without requirements for additional cycling (i.e., O divisions) (3). Thus from memory effectors IFN-γ can be detected within 6-24 h (7).

To establish whether the rapid IFN-γ induction by E75 is a property of existent activated CD8$^+$ cells and not the result of initiation of activation of resting cells, isolated CD8$^+$ cells from Donor 3 were stimulated with DC-E75 in the presence or absence of IL-12. Supernatants were collected 6 h later and analyzed for IFN-γ. Rapid induction of IFN-γ within 6 h was observed only in cultures containing E75+IL-12 **(Fig. 3A)**. The levels of IFN-γ continued to increase over the next 48 h. At this time, IFN-γ was detectable even from the cultures that did not receive IL-12, although its levels were significantly lower than in cultures that received IL-12. The levels of IFN-γ were dependent on Ag sequence, since FBP peptides, E39 and E41, mapping CTL epitopes showed similar (E41) or weaker (E39) abilities than E75 to induce IFN-γ. Secretion of IFN-γ was Ag concentration dependent, indicating that E75 specific T cells and not NK cells were the source of this cytokine. (not shown here, but presented from a separate experiment, **Fig 5**). Similar rapid IFN-γ induction by E75+IL-12 within 12h was observed with Donors 1 and 4 (not shown).

To verify that the IFN-γ induction in tumor Ag-reactive CD8$^+$ cells from PBMC is as rapid as the response of memory T cells reactive with conventional Ag, the experiment was repeated in the absence of IL-12 with CD8$^+$ cells from Donor 6, using E75, E39, and the AES peptide G76 as stimulators. The dominant HLA-A2 restricted CTL epitope from influenza matrix (M1:58-66) was used as positive control. A rapid IFN-γ response to M1, within 6-14h is commonly used to define the presence of activated memory cells to influenza in healthy individuals (7). All peptides were used at the same concentration. The results in **Figure 3B** show that in this donor E75, G76, and M1 at 50 µg/ml (~50 µM) E75, G76, and M1 induced IFN-γ within 20h even without exogenous IL-12. At 10 µg/ml none of these Ag induced detectable IFN-γ. The magnitude of response was Ag dependent. M1 induced the highest levels of IFN-γ. Tumor peptides induced IFN-γ from healthy donor CD8$^+$ cells but the IFN-γ levels were significantly lower than the ones induced by M1.

To establish that IFN-γ is induced in response to cognate Ag and confirm that the differences between the levels of IFN-γ are dependent on the Ag, the experiment was repeated with

Donor 5, using three HER-2 peptides (E75, GP2 and F57) reported to be recognized by CTL-TAL (10-12) and as control the unnatural Muc-1peptide D132. D132 was obtained by replacing Pro (P2, P4) with a P2 anchor (L) to ensure HLA-A2 binding and a charged residue in P4 (D) to perturb a TCR contact in the corresponding Muc-1 sequence. The HLA-A2 stabilizing ability of these peptides decreased in the order F57>D132>E75>GP2. The IFN-γ levels in the presence of IL-12 were: D132:105, F57:380, GP2:740, and E75:980 pg/ml, respectively. Since these differences were observed at the same Ag concentration, they likely reflect differences in peptide stimulatory potency and/or frequency of existent Ag-specific activated responders. Despite high background, the rapid IFN-γ response was always several folds higher for cognate HER-2 peptides, previously reported to be recognized by TIL/TAL than for the unnatural peptide D132. These results were confirmed with Donor 1: NP : 20pg, D132 : 25 pg, E75 : 160 pg. Thus peptides not present in the donor resulted in significantly lower reactivity.

To establish that the IFN-γ response to tumor Ag originated from memory cells, $CD8^+CD45R0^+$ and $CD8^+CD45R0^-$ cells were isolated from the same blood sample from Donor 3. Equal numbers of each population were tested in parallel for IFN-γ induction and proliferation in response to E75±IL-12 (**Fig. 3C, D**). The results show that $CD8^+CD45R0^+$ cells were the main producers of IFN-γ in response to E75 + IL-12. The levels of IFN-γ were by 5-fold lower when $CD8^+ CD45R0^-$ cells were used as responders. Comparison of the IFN-γ levels with the proliferative response demonstrated that E75 is a weak inducer of proliferation in both $CD45R0^+$ and $CD45R0^-$ cells. IL-12 did not synergize with E75 in increasing $CD8^+$ cells proliferation. Although the overall levels of 3H-TdR incorporation were higher in cultures, in the presence than absence of IL-12, the stimulation indexes for E75+IL-12-stimulated cultures were < 2.0. In the presence of IL-12, blocking of CTLA-4 in responders increased the IFN-γ response to E75 compared with E75+IL-12 by two fold. In the same experiment, blocking of CTLA-4 increased weakly the $CD8^+ CD45R0^+$ cells proliferation to E75+IL-12. This indicated that increased IFN-γ production it is not the result of E75+αCTLA-4 induced proliferation.

E75-dependent induction of endogenous IL-12 from DC. Synergy with IFN-γ. To elucidate the IFN-γ induction pathways activated by E75 we first determined whether IFN-γ induction required CD40L-CD40 interactions between APC and T cells. Isolated $CD8^+$ cells from Donor 3

were stimulated with 100 μg/ml E75 in the absence of IL-12. Usually at this Ag concentration IFN-γ could be detected in the absence of exogenous IL-12 (**Fig. 1C**). αCD40L and isotype control Ab were added to cultures stimulated in parallel, and the IFN-γ levels were determined at 24 and 48 h. The results (**Fig. 4A**) show that IFN-γ secretion was significantly inhibited in the presence of αCD40L suggesting that IFN-γ induction required CD40L-CD40 interactions between activated T cells and APC.

Since the CD40-CD40L interaction is the major pathway for T cell dependent IL-12 induction from APC, this raised the question whether IFN-γ induction is controlled through IL-12 induced from APC. To address this question we determined the effects of neutralizing IL-12 on IFN-γ induction. Parallel cultures were stimulated with E75 or M1 in the presence of a neutralizing α-IL-12 mAb, and an isotype control mAb (IC). E75 and M1 were used at 25 μg/ml. At this concentration E75 but not M1 required exogenous IL-12 to detect induced IFN-γ. The results in **Fig. 4B** show that IFN-γ production in response to both E75+IL-12 and M1+IL-12 was completely inhibited by α-IL-12, suggesting that induction of IFN-γ is dependent on IL-12.

To address whether E75-reactive CD8$^+$ cells induced IL-12 from DC, we determined the levels of IL-12 in the same experiment in response to E75 and control (no peptide). In addition, we tested in parallel whether IL-12 and IFN-γ are cofactors for IL-12 induction by E75. E75 rapidly induced IL-12 (**Fig. 4C**). Exogenous IL-12 had a modest synergistic effect with endogenous IL-12 in determining the overall IL-12 levels in the culture [240 pg endogenous + 360 pg exogenous = 600 pg, compared with 780 pg total detected]. This suggested that the co-stimulatory effect of exogenous IL-12 is not due to its own amplification. In contrast, IFN-γ at 50 pg/ml (the level induced by M1 in the absence of IL-12 in **Fig. 4B**) synergized with E75 in enhancing IL-12 levels: 240 + 50 = 290 pg vs 600 pg/ml. total detected (**Fig. 4C**, Column 3). Higher levels of IFN-γ were also detected in the wells stimulated with E75 + 50 pg IFN-γ, but not in the well stimulated with IFN-γ alone, demonstrating that IFN-γ can amplify its own response only in the presence of the tumor Ag (**Fig. 4D**). This effect does not require exogenous IL-12 because the levels of IL-12 induced by E75 + IFN-γ (shown in Fig. 4C) are above the threshold needed to costimulate IFN-γ induction.

These results suggest that E75-reactive CD8$^+$ cells induced IL-12, when recognized E75 on DC. Since IFN-γ was not detected at 25 µg E75 in this experiment, this suggested that compared with M1 the induced IL-12 levels were low and insufficient to co-stimulate IFN-γ. To address this question we determined in parallel the levels of IL-12 and IFN-γ induced in a concentration-dependent fashion by E75. The experiment was performed in the absence or presence of exogenous IL-12. We used M1 as a positive control. IL-12 production was Ag concentration dependent for both peptides (**Fig. 5A**). The levels of IL-12 induced by M1 were significantly higher than the levels induced by E75. Exogenous IL-12 did not change the dose-response pattern of IL-12 induced by the either Ag suggesting that within 24h it did not induce higher levels of IL-12 by itself.

The increase in overall levels of IL-12 following exogenous IL-12 addition was paralleled by increase in levels of IFN-γ in response to Ag. Thus the role of exogenous IL-12 is to compensate for the insufficient levels of IL-12 secreted by DC at encounter with tumor Ag reactive CD8$^+$ cells. This is also indicated by the fact that in the presence of IL-12 the molar ratios of IL-12: IFN-γ were similar for both peptides at both concentrations (**Fig. 5B vs. D**). The levels of IL-12 induced by 5 µg M1 were similar with the levels induced by 20 µg E75. At these peptide concentrations IFN-γ was not detected. Therefore, the results indicate that there is a minimum required level of endogenous IL-12 to be present in cultures for IFN-γ to be detected in response to tumor Ag. When IL-12 is below this level, IFN-γ cannot be detected in response to peptide stimulation (**Fig. 5A, C**). This deficiency was compensated sometimes by high concentrations (>100 µg/ml) of E75, (**Fig. 1E** and **Fig. 4A**), which induced substantially higher levels of IL-12 (>1600 pg/ml). (not shown)

Primary stimulation with E75 induced IL-2 in healthy donor CD8$^+$ cells. Enhancement by $\propto$B7.1. Regulation of T cell response by Ag involves at least two major mechanisms: the first by direct induction of IL-12 from APC, through CD40L-CD40 and the second through the B7-CD28 interaction (24, 25). The former apparently controls the IFN-γ induction, while the latter controls the IL-2 secretion and responsiveness to IL-2 through high affinity IL-2R induction (26, 27). The first pathway can also positively impact on the second through up-regulation, among others, of costimulatory molecules of the B7 family. A B7-CD28 dependent costimulatory pathway can also mediate a functional type 1 cytokine response (28, 29) and synergized with IL-12 (30). Results in **Fig. 1C and 3C** show that $\propto$-CTLA-4 enhanced IFN-γ induction in response to E75+IL-12. This suggested that the responders may be activated but negative signaling after ligation of B7 reduces

the response. If this is the case, blocking of B7 is expected to reverse the inhibitory effects. This will be evidenced by enhanced induction of IFN-γ, IL-2 and proliferation. To directly address the role of B7 in E75-induced cytokines, we investigated the role of B7-1 in IFN-γ and IL-2 induction.

To verify that induction of IFN-γ by E75 is enhanced by B7 blocking, the experiment was repeated with Donor 4 using isolated CD8$^+$ cells.   Since B7.1 and B7.2 were expressed on DC at different levels, we used alternatively $\propto$B7.1 and $\propto$B7.2 mAb to block the receptors ligation. In the absence of IL-12, $\propto$B7.1 did not enhance IFN-γ in response to E75, while $\propto$B7.2 co-stimulated IFN-γ induction only weakly ($\leq$50pg/ml). However, in the presence of IL-12, $\propto$B7.1 enhanced the E75+IL-12 induced response by two fold. The potentiating effect of $\propto$B7.2 was much weaker. The results (**Fig. 6A**) confirmed that the synergy between E75 and $\propto$B7 for high IFN-γ secretion within the first 24 h required IL-12. Since blocking of B7-CTLA4 synergized with Ag+IL-12 in IFN-γ production these results confirmed that the responders were activated CD8$^+$ cells.

To characterize the ability of E75-reactive CD8$^+$ cells to produce IL-2 the experiment was repeated with Donor 3 using M1 as positive control. Since in activated T cells blocking of B7/CTLA-4 was reported to reverse the state of tolerance of T cells for proliferation through induction of IL-2 (31), we investigated whether in our system $\propto$B7.1 was required for IL-2 induction in response to E75 and M1. E75 and M1 were used at the same concentrations (5 and 20 μg/ml) as for IL-12 and IFN-γ induction in the experiment shown in **Fig 5**. E75 induced IL-2 in a concentration-dependent manner in the absence of $\propto$B7.1. The IL-2 levels at 5 and 20 μg/ml E75 were 4 and 2 fold lower, respectively, than the levels induced by M1. 20 μg E75 induced the same levels of IL-2 as 5 μg M1. $\propto$B7.1 did not inhibit IL-2 production. In contrast it had a slight enhancing effect by 20% (E75) and 40% (M1) compared with peptide alone. Even in the presence of $\propto$B7.1 the levels of IL-2 induced by M1 were at least two fold higher than the levels induced by E75 (**Fig. 6B**). Thus previously in vivo activated CD8$^+$ cells are not tolerized/anergic with respect to IL-2 production.

Since IL-2 acts as a survival factor, while allowing production of endogenous IL-2, exogenous IL-2 at 200 pg/ml was added for the last 8h before supernatant collection in a parallel experiment performed simultaneously. The results in **Fig 6C** show the IL-2 levels induced by E75,

M1 ± αB7.1, after correction by subtracting the amount of exogenous IL-2 recovered in the control cells. The levels of IL-2 induced by E75, increased by two fold compared with the levels of IL-2 induced by E75 in IL-2 absence. In the same conditions the levels of IL-2 induced by M1 increased by 5 fold. αB7.1 increased significantly, by 2.5 fold, the levels of IL-2 induced by E75 but less, by 1.5 the levels of IL-2 induced by M1. The levels of IL-2 induced by 20 μg E75 + αB7.1 were similar with the levels induced by 5 μg M1. The IL-2 response in E75-stimulated cells was stable for the next 24h. At 48h, the IL-2 levels for 5 and 20 μg E75 were 88 and 120 pg/ml respectively. However, the response was short-lived and on day 5 less than 20 pg/ml IL-2 was detected in each well. Thus, when in vivo E75-activated cells remain viable they are able to produce more IL-2 in response to blocking of B7.1 confirming that they are not tolerized. However, while the 24h IL-2 response to E75 paralleled the IL-12 and IFN-γ response (**Fig. 5**), the IFN-γ production increased over the following 3 days, while the IL-2 secretion declined.

Priming with E75+αB7.1+IL-2 weakly increased CD8⁺ cells proliferation (**Fig. 7A**) similarly with E75+αCTLA-4 (**Fig. 3D**). In parallel, viable cells in cultures stimulated with E75+IL-2, and E75 +IL-2+αB7.1 increased by an average of 1.4 – 1.6 fold respectively compared with control cultures (not shown). This suggested that these cells undergo only limited clonal expansion in response to E75. Addition of E75+IL-2+αB7.1 to CD8⁺ cells from Donor 3 induced weak proliferation at 72h, although addition of IL-2+αB7.1 without E75 induced borderline proliferation compared with control E75-stimulated cultures: stimulation index (SI) = 2.53, compared with 1.28 and 1.0 respectively (993 cpm ± 50). Thus, in the presence of IL-2 with blocking of B7.1, CD8⁺ cells responded to the E75 signal better than in the absence of αB7.1. Since addition of αB7.1 increased IL-2 and only weakly proliferation, these results suggest that E75-stimulated cells *(a)* do not produce IL-2 in sufficient amount to support this response and *(b)* divide at slow rate. Thus the negative signaling through B7-CTLA4 could potentially provide a mechanism by which the proliferation of these cells is inhibited.

As expected, primary stimulation with DC-E75±IL-12, αB7/CTLA-4 did not induce Ag-specific cytolytic activity. Only occasionally Ag-specific CTL activity was observed in some donors (e.g. Donor 1 and 2) and was not enhanced at primary stimulation. (Anderson, Lee, Castelleja, preliminary data). Blocking of B7.1/CTLA-4 did not inhibit expression of cytotoxicity

in the donors where it was observed suggesting that DC-E75 priming in vitro did not activate naïve cells. This suggested that stimulation of these functions required different activation thresholds which cannot be reached by E75 in peptide form or at the concentrations used.

To address the possibility that the cells were not becoming nonresponsive as a result of activation, and required IL-12 as a consequence of tolerization, E75+IL-12 and M1+IL-12 primed cells from Donor 3 were restimulated with the corresponding peptides in the absence of IL-12. IFN-γ was determined 20h later. The results in **Fig. 7B** show that on a per cell basis these cells responded, in the absence of IL-12, with even higher levels of IFN-γ. Furthermore, to address whether they remain responsive to Ag with respect to clonal expansion, E75+IL-2 stimulated cells were washed on day 5, rested for 24h in the absence of IL-2 and restimulated with E75 on day 6. IL-2 at 20 pg/ml was added 24h later. The results in **Fig. 7C** show that restimulation with E75 of Donor 3 and Donor 5 CD8$^+$ cells increased the members of viable cells on day 12 by more than two-fold. Thus activated CD8$^+$ cells present in healthy donors became more responsive to restimulation through the TCR regarding proliferation and IFN-γ production.

Ag-specific CTL activity was enhanced at restimulation in only some donors (1, 5 and 6) but not in others (3 and 4). Ag-specific CTL activity was enhanced in Donor 3 at the third stimulation suggesting that differences in frequency together with the slow rate of division of these cells did not allow CTL effectors to reach the critical numbers for specific lysis to become evident. It should be mentioned that after 3-4 stimulations (days 21-25) the majority of cells (70%) began to undergo apoptotic cell death, and their viability could not be maintained by addition of IL-2, IL-7 and IL-15 alone or together. Thus it is possible that these cells have only a limited expansion potential, similarly with activated matrix specific memory cells (7).

These results show that activated tumor Ag reactive CD8$^+$ cells are present in healthy individuals and are not tolerized. They are responsive to stimulation through the TCR since they rapidly secrete IL-2, IFN-γ and IP-10 (Lee et. preliminary data) as well as induce IL-12. Tumor Ag such as E75, E41 or G76, are not tolerogenic but weak inducers of IL-2 and even weaker inducers of proliferation, suggesting that they are weak/partial agonists for activation of existent responders effector functions.

Discussion

In this report, we investigated the presence of activated, tumor Ag- and tumor-reactive cells in CD8$^+$ cells freshly isolated from PBMC of healthy donors. The IFN-γ, IL-2, and IL-12 induced by E75 and E75-reactive CD8$^+$ cells mirrored the response to M1 conventially used to define the presence of activated CD8$^+$ memory cells in healthy individuals. We found that E75–reactive CD8$^+$ cells are present in some donors in a state wherein they can secrete IFN-γ within 6-12h of antigen exposure. The frequent presence of activated memory cells reactive with tumor Ag and tumors in healthy individuals has not been previously reported. Our results show that T cells reactive with HER-2, FBP and AES peptides exist in healthy donors and they are rapidly activated by self-Ag (tumor Ag) in a similar fashion with the memory cells reactive with viral Ag. The fact that in the same donor IFN-γ was detectable at different time points in the absence or presence of IL-12 may reflect changes in the numbers of E75-specific cells or in their state of activation. Since the IFN-γ response was obtained primarily from CD45R0$^+$ cells, and was amplified by $\propto$CTLA4/$\propto$B7 only in the presence of IL-12, while the IL-2 response was amplified by $\propto$B7.1, our results suggest that E75-reactive nontolerized CD8$^+$ memory cells are frequently present in healthy individuals.

In all donors, high IFN-γ induction by Ag was detected within 20 h when costimulated by IL-12. The exogenous IL-12 requirement for detection of IFN-γ was dependent on the Ag sequence and concentration. The sequence dependency for peptides derived from the same protein was supported by the fact that in the same donor the IFN-γ responses to other HER-2 peptides of higher (F57) or weaker (GP2/F53) binding affinity to HLA-A2 were significantly lower than to E75. Thus, the higher or lower stabilizing ability of peptides from the same molecule did not enhance the "strength" (32) of the antigenic signal for IFN-γ induction suggesting that tumor Ag differed in TCR-stimulating potency at the concentrations used. This also raises the possibility that CD8$^+$ cells of different affinities for each tumor Ag and at different frequencies may be present in the same individual. This was suggested by the facts that: *(a)* IFN-γ responses to FBP peptide E41 and to the AES peptide G76 both of lower HLA-A2 affinity than E75 showed similar or higher IFN-γ levels with responses to E75; and *(b)* at high (100-150 µg/ml) E75 concentration exogenous IL-12 was not required for IFN-γ induction but was required when E75 was used at 5-20 µg/ml. Similarly, influenza-matrix, M1-reactive-CD8$^+$ cells from the same donor required exogenous IL-12 when

stimulated with 5 µg but not with 20 µg of M1. For both Ag (tumor and viral) the requirement for exogenous IL-12 inversely correlated with the amount of endogenous IL-12 induced.

The presence of activated CD8^{+} cells specific for E75 in the PBMC of healthy donors is also supported by the fact that these cells induced rapidly IL-12 from APC. IL-12 production increased in direct proportion to E75 concentration. Since induction of IL-12 require CD40 triggering, only when T cells are involved, (26) these results support the possibility that the responders to E75 and the other tumor Ag are activated CD8^{+} cells. Our results show that the amount of IL-12 induced in APC by peptide-reactive CD8^{+} cells should be above a certain level for IFN-γ to be detected in response to E75/M1. E75 at concentrations as high as 20 µM cannot induce sufficiently high levels of IL-12 required for mediation of IFN-γ costimulatory activity. The IL-12 dependent control of IFN-γ response to Ag may provide a mechanism for maintaining these cells in a non-responsive state. In the absence of exogenous IL-12, at the same Ag concentration, significantly higher levels of IL-12 were induced from the same APC by control M1 than by E75 reactive CD8^{+} cells (**Fig. 5**); In parallel, significantly higher concentrations of IFN-γ were induced by M1 than E75 from the same donor. These differences may reflect the requirement for higher levels of signaling by E75 and the other tumor Ag (AES, FBP) for TCR-mediated activation of the existent reactive CD8^{+} cells compared with conventional Ag. By comparing the levels of IL-12 and IL-2 induced by E75 and M1 at two Ag concentrations it appeared that M1 is at least 4-fold more potent than E75 in cytokine induction. Thus the lack of IFN-γ at low E75 concentrations did not reflect poor Ag presenting/T-cell-activating ability by DC used as APC.

To gain insight in the requirements for activation of these cells we investigated the mechanisms of activation that may be affected by E75 recognition. IL-12 production is amplified either by increased CD40L expression on T cells or by endogenous IFN-γ production. Both pathways are dependent on Ag concentration (26). Although E75 stimulation increased CD40L levels on T cells it did not increase the numbers of CD40L^{+} cells. Using two colors FACS analysis (CD40L-PE vs CD8-FITC) we found that at 20h E75 stimulation increased the vertical (Y) mean for CD40L^{+}CD8^{+} cells in the upper right quadrant by three fold (from 13.3 to 34.8). Addition of IL-12 doubled the Y mean level to 70.9, but not the % positive cells. The CD8^{+}CD40L^{+} cells were in the range 0.2-0.5%. We also determined the expression of IL-2R$\propto$ (CD25), an indicator of

responsiveness through TCR, on E75-stimulated cells. Stimulation with E75+IL-2 increased only weakly the Y mean for CD8$^+$ IL-2Rα^+ cells, from 23.2 in control (no peptide + IL-2) to 27.2 in E75+IL-2. Thus E75 appeared to have distinct potencies for induction of CD40L and IL-2Rα.

We demonstrated that IL-12 induction by E75 was not dependent on the presence of endogenous IFN-γ (**Fig. 5**). However, low (50 pg) of exogenous IFN-γ amplified the IL-12 response to E75. Therefore, low levels of IFN-γ induced by pathogens, or crossreactive Ag in the vicinity of these cells, in vivo may activate IFN-γ production by tumor Ag in these cells through a positive feedback loop: Ag1 + low IFN-γ $\rightarrow$ IL-12 $\rightarrow$ IL-2 + Ag2 $\rightarrow$ more IFN-γ. The exogenous IL-12 requirement for IFN-γ activation may be due to the fact that E75 is a weak inducer of CD40L. Low levels of CD40L in E75-reactive memory cells cannot induce the minimal levels of IL-12 to co-stimulate for IFN-γ production in response to E75. Another possibility which was not yet investigated but deserve attention in further studies is that the signal transduced by E75 in T cells is also a weak inducer of the IL-12Rβ2 chain, which is required for the high affinity IL-12Rβ expression. The high affinity IL-12R increase the sensitivity of responders to lower concentrations of IL-12, and it is stabilized by IFN-γ (33).

Given our data demonstrating a role for CD40L and IL-12 in regulating IFN-γ induction from E75-reactive CD8$^+$ cells and the reports that IFN-γ induction and IL-12 responsiveness in T cells can be also enhanced via CD28 we studied the involvement of B7-costimulation in IFN-γ and IL-2 production. The regulation of IFN-γ induction by IL-12 was confirmed by IFN-γ enhancement by mAb to B7/CTLA-4 only in the presence of IL-12. IL-12 production by the T cell dependent pathway did not require B7-CD28 interactions (26, 28). Since B7.1 and B7.2 were not upregulated on DC within 20h by interactions with T cells and cytokines, the ability of α-B7/CTLA-4 to amplify the IFN-γ response to E75 only in the presence of IL-12 suggest that these cells are activated but not anergic or anergized by negative signaling from TCR.

An enhancing role for blocking of B7 was observed in IL-2 induction. E75 alone induced IL-2 within 24h, although at low levels. α-B7.1 increased E75-induced IL-2. This increase was similar (1.25 fold vs 1.34 fold) with the increase mediated by αB7.1 on M1-induced IL-2. The fold increase was even higher for E75 than for M1 in the presence of IL-2: E75 + α-B7.1 vs E75 = 2.53,

while for M1+ ∝-B7.1 vs M1 = 1.47. Thus, the effects of ∝-B7.1 on IL-2 induction in both tumor and influenza matrix-reactive $CD8^+$ cells were similar at the same Ag concentration. This suggested that E75-reactive $CD8^+$ cells do not differ from positive control, in vivo activated matrix-reactive $CD8^+$ cells, in their ability to induce/secrete: IL-12, IL-2 and IFN-γ. They differed in the overall amounts of cytokines secreted. Since 4-fold more E75 was required to induce the same levels of IL-12, IFN-γ, and IL-2, induced by M1, this raises the possibility that the activated $CD8^+$ cells are in a hyporesponsive state.

Priming with E75 in the presence of IL-2, ∝CTLA-4 or ∝B7.1 although enhanced IL-2 production increased only marginally the responders, expansion and cytolytic activity. The reasons for this selective responsiveness are unknown. E75+IL-12 primed cells secreted high levels of IFN-γ and increased their expansion at restimulation with E75. Thus they maintained responsiveness through the TCR. However, their poor proliferative ability was not reversed by preculture in IL-2 as was expected if they were partially tolerized/anergic. Preculture and stimulation of these cells with 150 IU IL-2, IL-2+IL-15, RANTES, RANTES+IL-2, did not enhance E75-specific proliferation. The SI remained in the range 1.3 – 1.5 compared with controls (Lee, et. al preliminary data).

An alternative possibility is that this functional dichotomy reflects a weak agonistic activity of E75 in that the signal transduced through TCR can activate the IFN-γ, IL-12, IL-2 (this paper) and IP-10 induction (Lee et al. preliminary data) but sustains a slow division of these cells. The outcome of this slow division is that the frequency of specific cytolytic effectors increase slowly with each Ag stimulation. Based on $CD8^+$ $CD40L^+$/IL-2R∝$^+$ data the frequency of these cells is less than 10^{-3}. If a minimum frequency of $10^{-1} – 10^{-2}$ is required for detection of specific CTL activity (at E:T = 10-20/1) (7), and these cells increase in number by 4-5 fold after 2 stimulations, to reach the minimum threshold of 10 fold increase will require more than 3 stimulations. This is in agreement with reports on restimulation requirements for induction of tumor reactive CTL (34). In support of our conclusions, it has been recently shown that: (a) acquisition of cytotoxic function by activated CTL require at least one cell division (35) and (b) T cells that survive as memory cells proliferate weakly during the expansion phase of an immune response (36).

Our work raised the intriguing possibility that activated tumor Ag-specific CD8^{+} cells exist in vivo in healthy individuals. Their hyporesponsive state may be due to the increased threshold for self-reactivity as recently described in IEL from self-Ag^{+} mice (37). Alternatively, they were induced by another Ag (38), and the partial activation reflects cross recognition of the tumor Ag (38, 39). Epithelial tissues expressing HER-2 or FBP may provide the epitope precursors and/or crossreactive immunogens due to physiological turnover, while IL-12 induced by pathogens at the same time may costimulate in *trans* IFN-γ. Similarly, IL-2 from pathogen stimulated CD4^{+} cells can drive their expansion or act as survival factor. Activated CD8^{+} cells may not need professional APC for activation, since B7-CTLA4 interaction may be inhibitory. These populations may be maintained in steady-state by death of activated cells expressing higher-affinity receptors for Ag. The affinity for the tumor Ag of the surviving cells may decline over time. The surviving cells may be reactivated as TAL only when the tumor expresses very high concentrations of Ag (e.g. HER-2, gp100, FBP). At that time, tumor-derived IL-10, TGF, angiogenic chemokines, will inhibit the functional IL-12, IFN-γ and IP-10 response.

Identification of activated tumor-reactive memory CD8^{+} cells in healthy individuals with high frequency raise several novel and possibly important implications for tumor immunosurveillance, and vaccine design: *(a)* since such cells are present in both healthy donors and cancer patients, their rapid mobilization to mediate cytokine associated effector functions may be useful for maintaining a mechanism of control of tumor emergence in patients with no evidence of disease. This may be achieved by periodic stimulation with low concentrations of tumor Ag plus IL-12. This should be particularly relevant for ovarian cancer where the recurrence rate is high. *(b)* The sensitivity of IL-12 induction and of the IL-12R to negative regulation by IL-10, may require, in cancer patients, approaches to neutralize regulatory cytokines if the activation of IFN-γ^{+} effectors is aimed (2). *(c)* Naïve CD8^{+} cells primed by tumor Ag plus B7 costimulation, over time convert to memory cells. After death, due to repeated encounters with Ag, in vivo, the surviving cells of lower affinity for Ag will require even higher Ag concentrations for activation than at priming, theretofore blocking of . $\propto$B7/CTLA4 will be unable to reverse negative regulation of CTL expansion if the tumor Ag cannot reach the threshold for TCR signaling for cell cycling, even if exogenous IL-2 is provided as a growth survival factor. *(d)* The weak signaling demonstrated by the wild-type tumor Ag would require development of TCR agonists specific for activation of proliferation to be used

for vaccination. Design of functional agonists of E75 and E39 is in progress in our laboratory. Therefore, the results reported above may be useful to develop approaches to activate cellular immunity to tumors.

Figure Legends

Figure 1. Freshly isolated, unstimulated plastic non-adherent PBMC from healthy donors (A, B, D, E) and breast cancer patients (C) specifically secrete IFN-γ within 20h in response to E75. (A) Donor 1. PBMC were collected on days 0, 120, and 330 counting as Day 0 the date of first collection and stimulated with E75 at 50 µg/ml. (-) indicate not tested. (B) Donor 2. E75 at 50 µg/ml. (C) Unimmunized breast cancer patient. Supernatants were collected at 24h after stimulation with 50 µg/ml E75. IL-12 was used at 3 U (330 pg/ml). (D) Donor 3. Days 7 and 14 indicate the days after the first stimulation when the experiment was repeated with fresh PBMC. Day 1: 50 µg/ml E75 + 330 pg/ml IL-12; Day 7: 25 µg/ml E75 + 150 pg/ml IL-12; Day 14: 20 µg/ml E75 + 150 pg/ml IL-12. (E) Donor 5: 100 µg/ml E75 + 330 pg/ml IL-12.

Figure 2. (A) Freshly isolated T cells from Donor 1 produce higher levels of IFN-γ within 24 h of co-culture with SKOV3.A2 compared with SKOV3. (B) IFN-γ response to SKOV3.A2 is dependent on HLA-A2 recognition. Experimental conditions as described in the Materials and Methods.

Figure 3. (A) Induction of IFN-γ by E75 within 6 h □, and 48h □ from isolated CD8$^+$ cells from Donor 3. All peptides were used at 10 µg/ml; IL-12 at 330 pg/ml. (B) IFN-γ induction by tumor peptides E75, E39, G76 and positive control M1 within 20 h in the absence of exogenous IL-12 is Ag and concentration dependent. (C) IL-12 and ∞CTLA-4 mAb synergize in enhancing IFN-γ induction in response to E75 in isolated CD8$^+$ CD45RO$^+$ cells. Equal numbers (10^6) of CD8$^+$ CD45RO$^+$ and CD8$^+$ CD45RO cells were used as responders in each well. (D) E75 in the presence of IL-12 and ∞CTLA-4 enhanced only marginally CD8$^+$ cells proliferation. Equal numbers of CD8$^+$ CD45RO$^+$ and CD45RO$^+$ depleted (CD45RO$^-$) were collected from cultures after 48 h and used to determine differences in the rate of proliferation by E75. 10^5 Donor 3 live cells were incubated with of ^{3}H–TdR for 8 h. The experiment was performed in tetraplicate. Differences in ^{3}H–TdR are significant for all the ($\pm$) E75 groups by the Student's t–test, but the stimulation indexes (SI) are $\leq$ 2.0.

Figure 4. Induction of IFN-γ from E75-reactive CD8$^+$ cells requires CD40L-CD40 interactions and is controlled by IL-12. (A). Isolated CD8$^+$ cells from Donor 1 were stimulated with 100 µg/ml E75 in the presence of αCD40L mAb (Δ) or isotype control mAb ($\square$). ($\circ$) indicates background levels from cultures not stimulated with peptide (B). The same responders were stimulated with E75 and M1 at 25 µg/ml in the absence (-) or presence (+) of IL-12 (330 pg/ml). α-IC indicate isotype control Ab. (C,D). In a separate experiment responders were stimulated with E75 at 25 µg/ml in the absence(-) or presence(+) of IL-12 (330 pg/ml) or IFN-γ (50 pg/ml). NP indicate no peptide.

Figure 5. Concentration-dependent induction of IL-12 (A,B) and IFN-γ (C,D) by E75 and M1. $\pm$IL-12 indicate that exogenous IL-12 was not added (-) or used (+) at 330 pg/ml. IL-12 and IFN-γ were determined from the same experiment from the same supernatants collected 20 h after stimulation. (O,$\square$) E75, (Δ, $\bullet$) M1.

Figure 6. (A) The synergy between E75 and αB7.1/B7.2 mAb in enhancing IFN-γ requires the presence of IL-12. In the absence of IL-12, low levels of E75 induced IFN-γ were detected in the presence of αB7.1 at 48 h (50 pg/ml). (B,C) E75 synergize with α B7.1 in enhancing IL-2 induction. Exogenous IL-2 was added at 200 pg/ml. 8h before supernatant collection. C. Results indicate pg/ml IL-2 after subtracting the levels of IL-2 resolved in the control well (179 pg). Both peptides were tested in the same experiment performed in parallel. IL-2 was determined in the same experiment. (A, B, C) Donor 3 isolated CD8$^+$ cells. αB7.1 and B7.2 mAb were added to the DC 1 h before addition of responders. $\square$ indicates levels of IL-2 determined at 24h from cultures stimulated with either peptide at 20 µg/ml in the presence of αB7.1.

Figure 7. (A) Proliferative responses of CD8$^+$ populations. αB7.1 enhanced the rate of proliferation induced by priming with E75 in the presence of IL-2. CD8$^+$ cells from Donor 3 were stimulated with DC-E75 for 72h. 10^5 cells were collected from each well, and pulsed with 3H-TDR for the last 8h. All measurements were performed in tetraplicate. Results indicate cpm/50,000 cells. $\pm$ SD. (B) IFN-γ response of preactivated Donor 3 CD8$^+$ cells. CD8$^+$ cells primed with DC-E75 +IL-12 and maintained in culture for a total of 5 days were rested for 24h in the absence of IL-2 and restimulated on day 6 with DC-E75 without exogenous IL-12. Supernatants were collected at

20h. Results indicate pg/ml/10^6 cells. (C) CD8$^+$ cells from Donor 3 were primed with DC-E75 (●) or DC-NP (O) on day 0 as described in Material and Methods. Live cells were counted on day 6, washed, and restimulated in the same conditions with DC-E75 followed by IL-2 16h later. (▲) Donor 5 stimulated with DC-E75. Results indicate live cells/ml x 10^{-6}.

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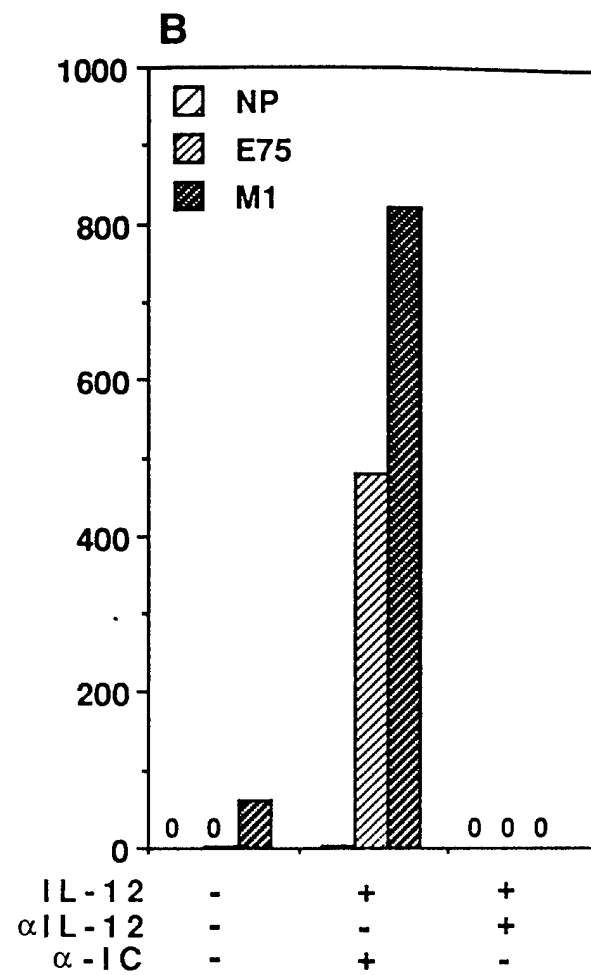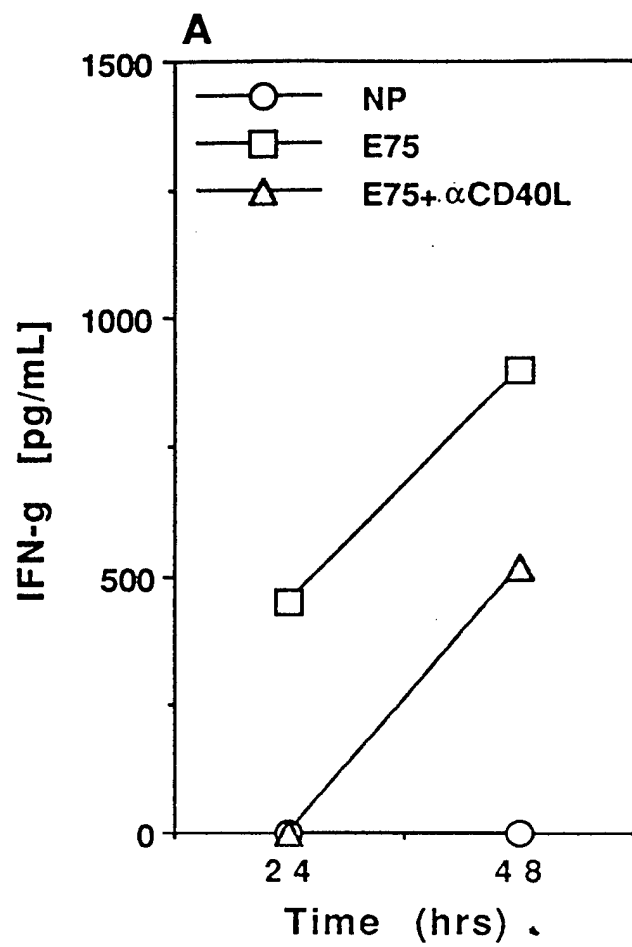
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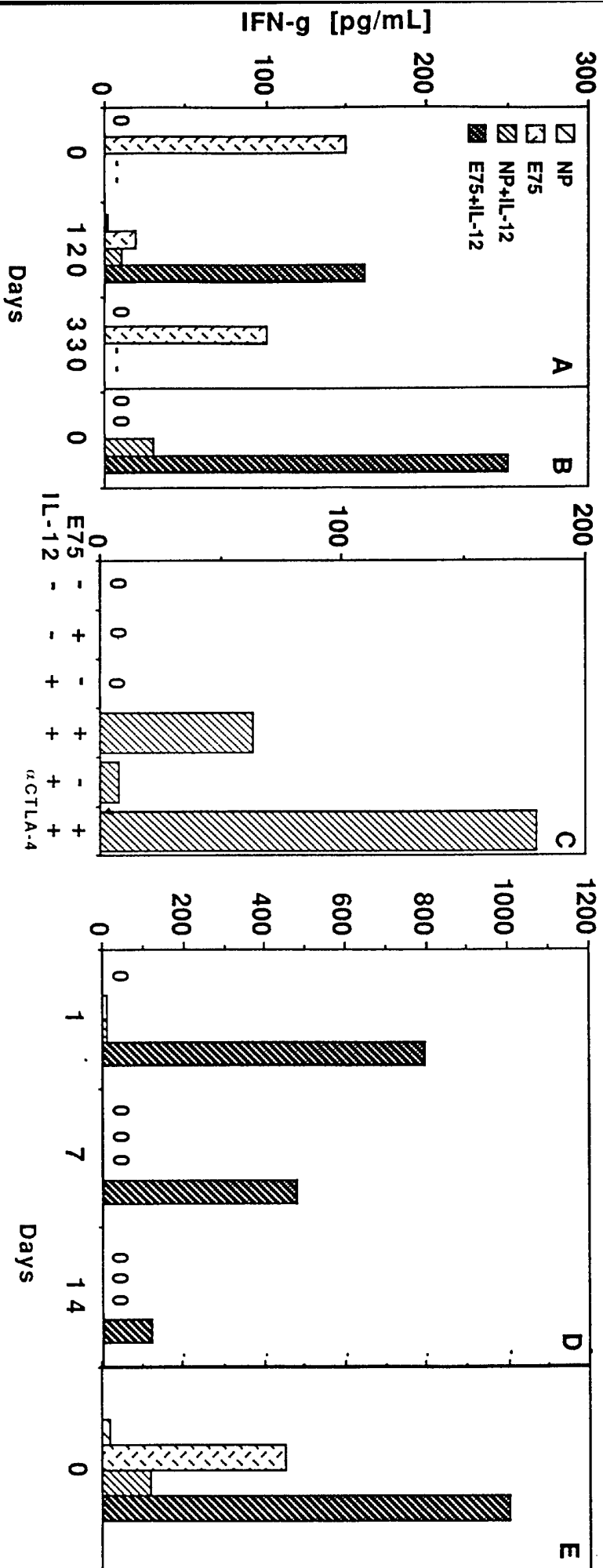
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